



## Original article

# Systemic TNF- $\alpha$ blockade attenuates anxiety and depressive-like behaviors in *db/db* mice through downregulation of inflammatory signaling in peripheral immune cells

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## ARTICLE INFO

## Article history:

Received 20 February 2020

Accepted 2 April 2020

Available online 9 April 2020

## Keywords:

*db/db* mice

TNF- $\alpha$

Etanercept

Inflammation

Obesity

Depression

Anxiety-like behavior

Depressive-like behavior

## ABSTRACT

Research studies have indicated that the comorbidity burden of mood disorders and obesity is reasonably high. Insulin signaling has been shown to modulate multiple physiological functions in the brain, indicating its association with neuropsychiatric diseases, including mood disorders. Leptin is a hormone responsible for regulating body weight and insulin homeostasis. Previous studies on *db/db* mice (a mouse model that carries a spontaneous genetic mutation in leptin receptor *Lepr<sup>db</sup>*) have shown that they exhibit inflammation as well as neurobehavioral traits associated with mood. Therefore, targeting inflammatory pathways such as TNF- $\alpha$  may be an effective strategy in the treatment of obesity-linked mood disorders. The objective of this study was to investigate the effect of long-term administration of etanercept (a TNF- $\alpha$  blocker) on anxiety and depressive-like behaviors in *db/db* mice. This was performed using light/dark box, forced swim, and open field tests with lean littermate wild type (WT) mice serving as a control group. Using flow cytometry in peripheral blood, we further examined the molecular effects of etanercept on NF- $\kappa$ B p65, TNF- $\alpha$ , IL-17A, and TLR-4 expressing CD4+, CD8+, and CD14+ cells in the peripheral blood. Our data show that peripheral administration of etanercept decreased these cells in *db/db* mice. Furthermore, our results indicated that peripheral administration of etanercept reduced anxiety and depressive-like behaviors. Therefore, targeting TNF- $\alpha$  signaling might be an effective strategy for modulating obesity-associated depression and anxiety.

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## 1. Introduction:

There is a high prevalence of comorbidities associated with obesity, including neuropsychiatric disorders such as depression and anxiety (Brumpton, Langhammer, Romundstad, Chen & Mai, 2013; Dawes et al., 2016). Type-2 diabetes mellitus (T2DM) or non-insulin dependent diabetes mellitus is a disease characterized

by chronic hyperglycemia, insulin resistance, and obesity. These symptoms can result in microvascular complications that impact multiple organs and tissues, including the eyes, kidneys, and peripheral nerves (Malik, Tesfaye & Ziegler, 2013). Persistent hyperglycemia causes peripheral oxidative stress and inflammation, both of which are associated with cardiovascular complications, a decline in cognitive function, anxiety, and depression (Vincent, Callaghan, Smith & Feldman, 2011; O'Brien et al., 2015; Wang, Chandrasekera & Pippin, 2014). Over the past few decades, considerable resources have been devoted to T2DM research using different animal models, such as the leptin receptor-deficient *db/db* mouse model (*Lepr<sup>db/db</sup>* mice) (Martinez-Botas et al., 2000; O'Brien et al., 2015).

Previous research employing forced swim and open field tests with *db/db* mice has shown that they exhibit anxiety and depressive-like behaviors. However, the long-term pharmacologi-

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Peer review under responsibility of King Saud University.



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cal administration of rosiglitazone, an antidiabetic agent, altered these phenotypes. This suggests that the management of blood glucose in a T2DM animal model may reduce the co-occurrence of anxiety and depressive-like behaviors (Sharma, Elased & Lucot, 2012).

Recent studies have indicated an essential role for innate and adaptive immune cells such as macrophages and T cells in the release of inflammatory mediators located within adipose tissue (Weisberg et al., 2003); most notably, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) level (Mantzoros et al., 1997). TNF- $\alpha$  plays a vital role in the progression of cognitive decline, as well as depressive and anxiety disorders (Bai, Chiou, Su, Li & Chen, 2014). Similarly, IL-17A has been reported to be crucial in mediating inflammatory processes associated with CNS disorders, including anxiety and depression (Beurel, Harrington & Jope, 2013; Waisman, Hauptmann & Regen, 2015).

Neuroinflammation is associated with obesity as well as depression (Wang, Xu, Liu, Li & Li, 2018). The toll-like receptor 4 (TLR-4) is expressed on circumventricular organs and the choroid plexus, and is linked with NF- $\kappa$ B activation and subsequent production of TNF- $\alpha$  (Nadeau & Rivest, 2000). Augmented TLR-4/NF- $\kappa$ B signaling has been reported in stressful conditions, indicating a possible functional association with the pathophysiology of depression (Rethorst, Bernstein & Trivedi, 2014). NF- $\kappa$ B activation induces cytokine expression and regulates the inflammatory cascade (Li et al., 2013). It was also found to be associated with increased TNF- $\alpha$  expression in the hippocampus and frontal cortex in a chronic mild stress animal model (Jiang et al., 2013; Wang et al., 2018).

Etanercept is one of the most widely used anti-TNF- $\alpha$  agents and has been approved for the treatment of a variety of inflammatory diseases including rheumatoid arthritis, ankylosing spondylitis, psoriasis, and psoriatic arthritis (Caporali et al., 2009). Recent studies have shown that the administration of etanercept reduces anxiety and depressive-like behaviors in rodents (Bayramgürler, Karson, Özer & Utkan, 2013; Camara et al., 2014). Inflammatory cytokines have the potential to induce sickness behavior that may later develop into depression-like symptoms (Adzic et al., 2018; Dantzer, O'Connor, Freund, Johnson & Kelley, 2008). Furthermore, inflammatory cytokines such as IL-6, IL-1 $\beta$ , and TNF- $\alpha$  have been found to be elevated in patients with depression. This suggests there is bidirectional communication between the peripheral inflammation and the central nervous system (Adzic et al., 2018; Dowlati et al., 2010).

In the present study, we examined whether *in vivo* blocking of peripheral TNF- $\alpha$  signaling would improve anxiety/depression-like behavior in *db/db* mice. Our data show that the peripheral administration of etanercept reduced anxiety and depressive-like behaviors, which was associated with a concomitant reduction in peripheral inflammation. This suggests that the peripheral administration of an anti-inflammatory agent could be useful in the modulation of obesity-associated anxiety and depressive-like behavior.

## 2. Materials and methods

### 2.1. Chemicals and antibodies

Etanercept was purchased from Enbrel (Enbrel<sup>®</sup>, MyClic-Germany). Heparin was purchased from Sigma-Aldrich, USA. Fluoroisothiocyanate (FITC), Phycoerythrin (PE), Allophycocyanin (APC) labeled and PE/Dazzle-labeled CD4, CD8, CD14, TLR-4, IL-17A, NF- $\kappa$ B, and TNF- $\alpha$  anti-mouse monoclonal antibodies, FcR blocking reagent, RBC's lysing, fixation, and permeabilizing buffers were purchased from Miltenyi Biotec, Germany; BioLegend, Santa Cruz, Dallas; and BD Biosciences.

### 2.2. Animals

Adult male *db/db* mice (six-eight weeks old; Strain B6.BKS (D)-*Lepr<sup>db</sup>/J*, Stock No. 000697 B6 db) and age-matched non-diabetic lean control mice (littermate wild type) were included in the study. They were bred in the KSU animal care facility by mating heterozygous (*Lepr<sup>db/+</sup>*) males and females. Mice genotypes were confirmed by tail tissue and PCR analysis conducted by Transnetyx, Inc (Cordova, TN, USA). The mice were maintained at a room temperature of 22 °C  $\pm$  2 °C with a 12:12-h light/dark cycle and 40 to 60% humidity. They were housed in a specific pathogen-free environment, fed with standard rodent chow, and provided with water *ad libitum*. All experimental procedures were performed in accordance with the guidelines of the Institutional Research Ethics Committee (REC), King Saud University. To confirm the presence of diabetes in the models, the mice were made to fast for 6 h. Their blood glucose levels were then measured using 1–2 drops of blood collected from their tails on a glucometer chip (Accu-Check Advantage Blood Glucose Monitor, Roche Diagnostics Corp., Indianapolis, IN, USA). All *db/db* mice with a fasting blood glucose level >200 mg/dl were considered diabetic and included in the study (data not shown).

### 2.3. Experimental design and drug administration

The mice were acclimatized for two weeks and divided randomly into the following four groups: (1) control lean mice (littermate WT) given normal saline intraperitoneally (i.p.); (2) *db/db* mice + normal saline; (3) littermate WT mice + etanercept; and (4) *db/db* mice + etanercept. In line with Chio et al. (2013), a 5 mg/kg (i.p.) dose of etanercept was administered once every other day for 21 days. Each group consisted of six mice, and the amount administered was based on the body weight of each mouse. Following treatment, behavioral studies were conducted, after which the mice were anesthetized using a mixture of ketamine (50 mg/ml) (Tekam) and xylazine (20 mg/ml) (Seton) (dose 0.1 ml/10 gm i.p.). Mice blood was collected from the retro-orbital sinus.

### 2.4. Locomotor activity

Locomotor activity was determined using a Supermex apparatus, comprising a floor area 26  $\times$  26 cm<sup>2</sup> in size and a sensor monitor mounted above the chamber (Muromachi Kikai, Tokyo, Japan). As described in previous research (York, Blevins, McNeil & Freund, 2013), each mouse was kept in the Supermex apparatus for 10 min prior to the locomotor activity test to habituate them to the environment. They were then kept for another 10 min in the apparatus to record their locomotor activity. This was measured using activity-monitoring software (Any-Maze). All locomotor activity (distance traveled) was automatically scored and summed and the cut-off time for the test session was 1 min.

### 2.5. Open field test

An open field test was used to measure the anxiety-like behavior and locomotion of the mice, as described in previous research (Pietropaolo, 2010). All mice were carried to the test room in their home cages. Each mouse was housed in the apparatus 10 min prior to the open field test to habituate them to the environment. They were then placed in the center of the open field test apparatus and allowed to explore for 5 min, after which time they were returned to their cages. The cumulative time spent in corners by each mouse was calculated during the test period to assess anxiogenic behavior. The open field was then cleaned with 70% ethyl alcohol and permitted to dry between tests.

## 2.6. Light/dark exploration test

Light/dark (L/D) was used to assess anxiety-like behavior. The test apparatus consisted of a  $42 \times 21 \times 25 \text{ cm}^3$  box that was equally divided into light and dark compartments. The opening between these compartments was 4 cm wide and 3 cm high. As described in previous research (Bourin & Hascoët, 2003), each mouse was placed in the light compartment at the start of the experiment and its behavior examined for a period of 5 min. The cumulative time spent in the dark compartment by each mouse was calculated during the test period to assess anxiogenic behavior.

## 2.7. Forced swim test

In the forced swim test (FST), cylindrical Plexiglas water tanks  $24 \text{ cm} \times 18 \text{ cm}$  in size were used. Each tank contained sensors, a mercury thermometer, a paper towel, and heating pads. We used the Forced swimming test system (CompACT FSS ver. 2) to record data for a six-minute test session. The time spent immobile within the final 4 min was used to assess the depressive-like behavior of mice (Yankelevitch-Yahav, Franko, Huly & Doron, 2015).

## 2.8. Flow cytometric analysis

Blood was collected in heparinized tubes from the retro-orbital plexus to assess CD4, CD8, CD14, and TLR-4 surface markers. FITC- or PE-labeled CD4, PE- or FITC-labeled CD8, APC-labeled TLR-4, and PE/Dazzle-labeled CD14 (BioLegend, USA) monoclonal antibodies were used to determine the percentage of CD4<sup>+</sup>, CD8<sup>+</sup>, TLR-4<sup>+</sup>, and CD14<sup>+</sup> cell surface receptors, respectively. These were added to 100  $\mu\text{L}$  of blood lysed with a lysing buffer (BioLegend, USA). Approximately 20  $\mu\text{L}$  of the fluorescently labeled monoclonal antibodies were added to the suspension of blood leucocytes (CD4 FITC, CD8 PE, CD14 PE/Dazzle-labeled, and TLR-4 APC, BioLegend, USA). These were then incubated at room temperature for 30 min. To stain the intracellular markers IL-17A, NF- $\kappa\text{B}$  p65, and TNF- $\alpha$ , the cells were permeabilized, subjected to fixation by standard buffers (BioLegend, USA), and stained with intracellular APC-IL-17A, FITC-NF- $\kappa\text{B}$  p65, and PE-TNF- $\alpha$  antibodies (BioLegend, USA; Santa Cruz, Dallas, USA), (Ahmad, Attia, Zoheir, Ashour & Bakheet, 2014). After centrifugation at 300g for 10 min, the samples were kept at 4 °C until flow cytometry analysis was conducted. Immunostained leukocytes in each sample were acquired using an FC 500 flow cytometer; 10,000 events/sample were then analyzed using CXP software (Beckman Coulter, USA).

## 2.9. Statistical analysis

Data are presented as mean  $\pm$  SEM. Data were analyzed using a one-way ANOVA with Dunnett's *post hoc* test for multiple comparisons on GraphPad Prism 5 (GraphPad Software, San Diego, USA). The level of statistical significance was set at  $p < 0.05$ .

## 3. Results

### 3.1. Etanercept reversed anxiety-like behavior in *db/db* mice

Because obesity is linked with anxiety and depression, we examined whether the reduction in obesity-associated inflammation would ameliorate anxiety like-behavior in *db/db* mice. Our data show there was an increase in anxiety-like behavior in these mice, as reflected by a significant decrease in locomotor activity (Fig. 1A). Furthermore, time spent in the corner by *db/db* mice significantly increased during the open field test (Fig. 1B). However,

there was no significant difference between *db/db* and littermate WT mice in their exploration of the dark area in the light-dark box (Fig. 1C). These observations reversed significantly in the open field test following etanercept treatment in *db/db* mice, and there was an upward trend in the locomotor activity test. Notably, the administration of etanercept did not affect the performance of treated littermate WT mice compared with the control group (normal saline group; Fig. 1). These results suggest that the inhibition of TNF- $\alpha$  signaling could be responsible for the reversal of obesity-induced behavioral changes. This indicates that a functional link may exist between obesity, inflammation, and anxiety-like phenotypes.

### 3.2. Etanercept reduced depressive-like behavior in *db/db* mice

We also examined depressive-like behavior using the forced swim test. The results indicate that the administration of etanercept reduced immobility time in etanercept-treated *db/db* mice (Fig. 2).

### 3.3. Etanercept reversed the increase in IL-17A<sup>+</sup> and IL-17A-producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells peripherally in *db/db* mice

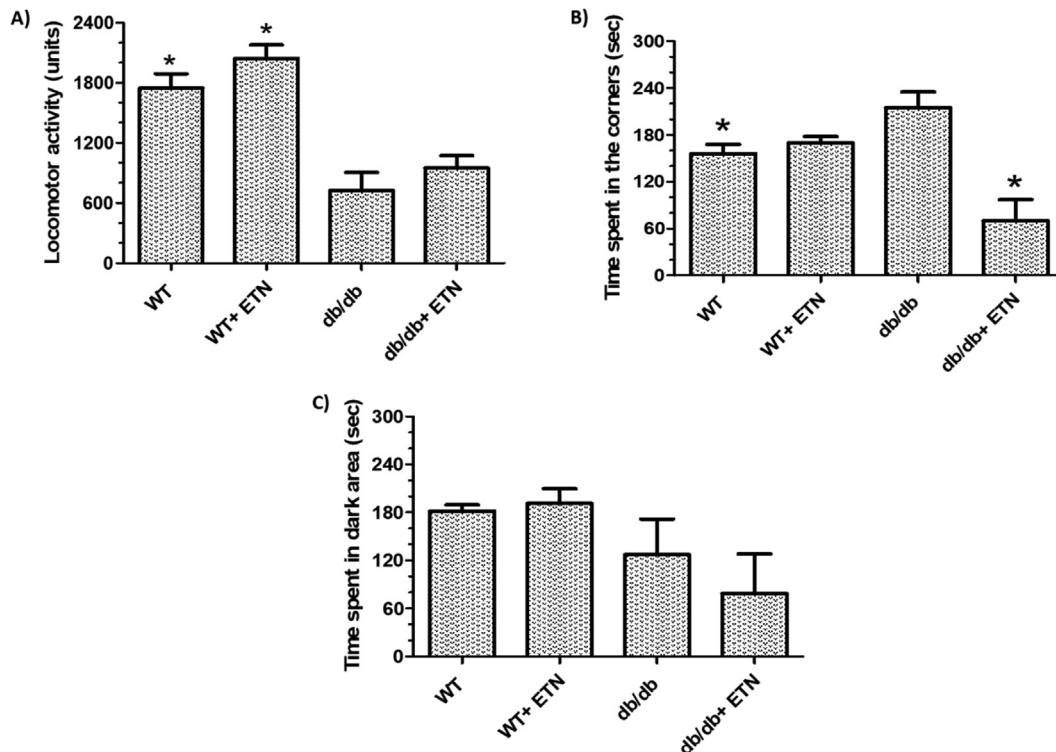
Because IL-17A has been linked with both anxiety and depressive disorders, we evaluated the effect of TNF blockade on IL-17A expression in CD4<sup>+</sup> and CD8<sup>+</sup> T cells of peripheral circulation. Our data showed significantly elevated IL-17A expression in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells of untreated *db/db* mice compared with untreated littermate WT mice (Fig. 3A and B). However, there was significant attenuation of IL-17A expression in CD4<sup>+</sup> and CD8<sup>+</sup> T cells in etanercept-treated *db/db* mice compared with untreated *db/db* mice (Fig. 3A and B), as reflected by dot plots in Fig. 3C and D, respectively.

### 3.4. Etanercept reduced NF- $\kappa\text{B}$ p65 in *db/db* mice

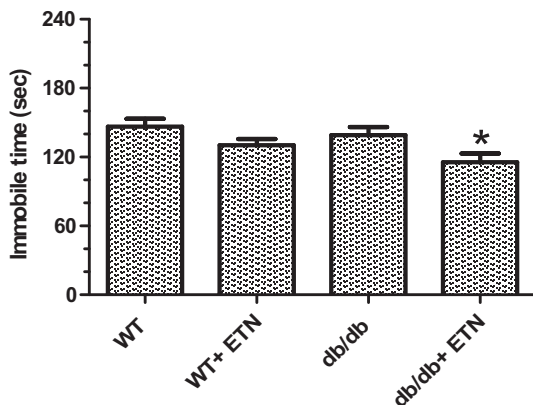
We further explored the effect of etanercept on NF- $\kappa\text{B}$  p65 in *db/db* and littermate WT mice. We found higher levels of NF- $\kappa\text{B}$  p65<sup>+</sup> and NF- $\kappa\text{B}$  p65<sup>-</sup> expressing CD4<sup>+</sup> and CD8<sup>+</sup> T cells in untreated *db/db* mice than in littermate WT mice (Fig. 4A and B). By contrast, when *db/db* mice were treated with etanercept, there was a significant decrease in NF- $\kappa\text{B}$  p65<sup>+</sup> and NF- $\kappa\text{B}$  p65<sup>-</sup> expressing CD4<sup>+</sup> and CD8<sup>+</sup> T cells compared with untreated *db/db* mice (Fig. 4A and B), as reflected by dot plots in Fig. 4C and D respectively. Etanercept-treated *db/db* mice also exhibited significantly decreased NF- $\kappa\text{B}$  p65 expression compared with untreated *db/db* mice (Fig. 4A).

### 3.5. Etanercept normalized TNF- $\alpha$ in *db/db* mice

We further assessed the effect of etanercept on the expression of TNF- $\alpha$  in peripheral blood. As shown in Fig. 5A, there was a higher level of TNF- $\alpha$  -producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells in untreated *db/db* mice than in littermate WT mice. Moreover, *db/db* mice treated with etanercept exhibited a significant decrease in TNF- $\alpha$  -producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells compared with untreated *db/db* mice (Fig. 5A and B), as reflected by dot plots in Fig. 5C and D, respectively. Together, these results suggest that inflammatory signaling was increased peripherally in *db/db* mice. This could be a factor contributing to the anxiety/depressive-like behaviors observed in *db/db* mice and can be attenuated by treatments with anti-inflammatory agents.



**Fig. 1.** Anxiety-related behavioral tests in *db/db*, littermate WT mice, and Etanercept-treated mice. (A) Locomotor activity test, (B) Open field test, and (C) Light/dark box test. The four groups of mice were: littermate WT + saline, *db/db* + saline, littermate WT + 5 mg/kg ETN, and *db/db* mice + 5 mg/kg ETN; i.p. an injection every other day for 21 days. \* $P < 0.05$  compared with *db/db* saline-treated mice. Data are shown as mean  $\pm$  SEM ( $n = 6$ ). Littermate WT = lean control mice, ETN = etanercept treated mice.



**Fig. 2.** Results of a forced swim-test to examine depressive-like behavior in four groups of mice: littermate WT + saline, *db/db* + saline, littermate WT + 5 mg/kg ETN, and *db/db* mice + 5 mg/kg ETN; i.p. an injection every other day for 21 days. \* $P < 0.05$  compared with littermate WT saline-treated mice. Data are shown as mean  $\pm$  SEM ( $n = 6$ ). Littermate WT = lean control mice, ETN = etanercept treated mice.

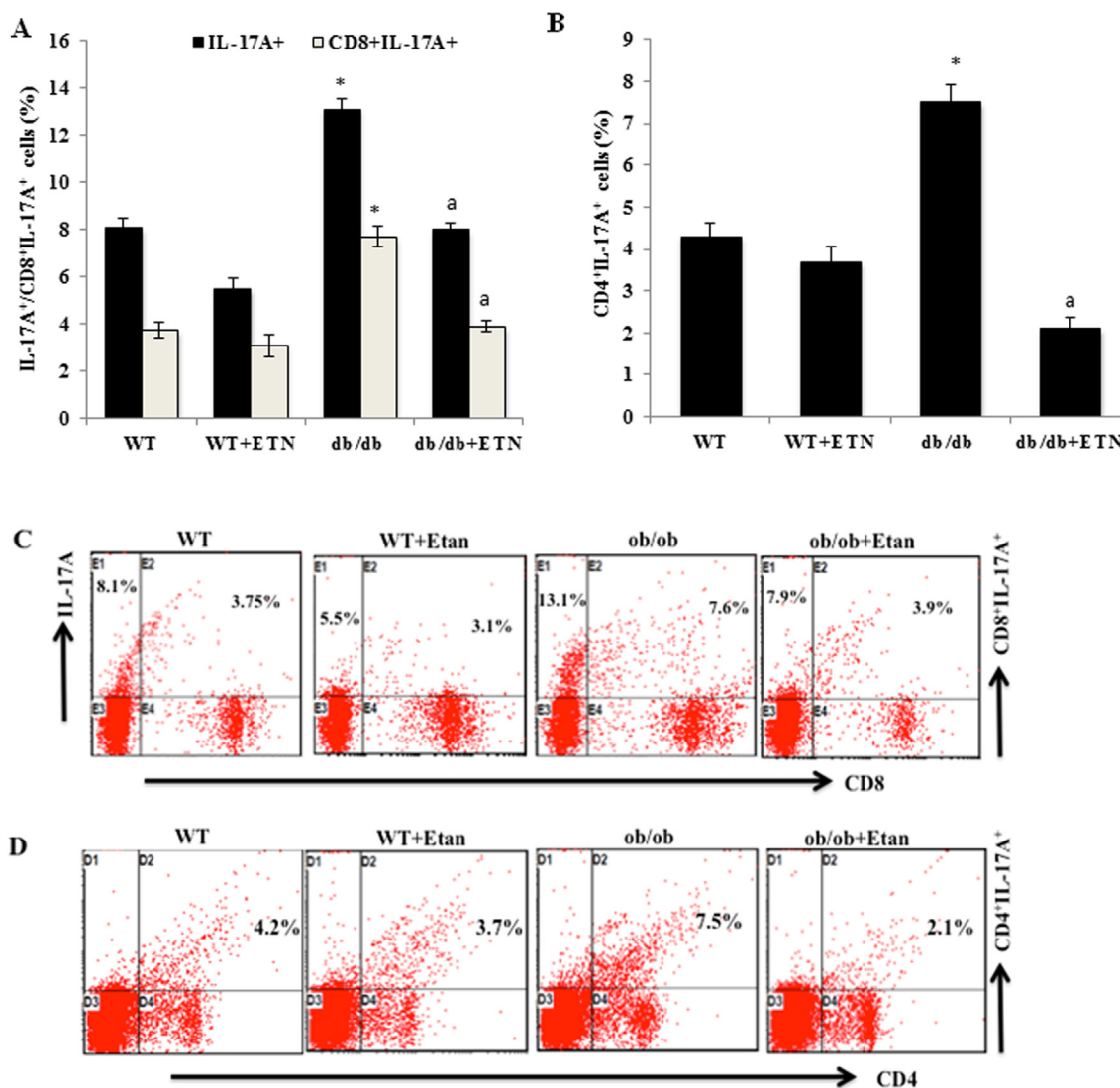
### 3.6. Etanercept mitigated TLR-4 signaling

We further investigated the effect of etanercept on TLR-4-expressing CD14<sup>+</sup> cells. Untreated *db/db* mice exhibited an increase in TLR-4-expressing CD14<sup>+</sup> cells in the peripheral blood compared with untreated littermate WT; this was significantly mitigated by the administration of etanercept (Fig. 6A). These results suggest that obesity-linked inflammation plays an essential role in the upregulation of TLR-4 in the periphery of *db/db* mice. Blocking TNF- $\alpha$  signaling could therefore be responsible for the amelioration of anxiety/depressive-like behavior as it reduces TLR-4 signaling.

## 4. Discussion

The main objective of this study was to examine whether a reduction in systemic inflammation using etanercept (a TNF- $\alpha$  inhibitor) has the potential to improve obesity-associated mood dysregulations such as anxiety/depression. Our results showed that systemic treatment with etanercept reduced both anxiety and depressive-like behavior as well as peripheral inflammation in *db/db* mice. This suggests there is a strong link between obesity, inflammation, and mood disorders.

Stress could be a significant contributory factor in the development of diabetes and metabolic disorders (Castaneda et al., 2011; Trento et al., 2010). A preclinical post-traumatic stress disorder study indicated a significant impairment in glucose tolerance (Castaneda et al., 2011; Lin et al., 2004). The prevalence of depression in diabetic patients is approximately double that of non-diabetic individuals (Anderson, Freedland, Clouse & Lustman, 2001). Previous studies have established a link between chronic systemic inflammation, diabetes, insulin resistance, and major depressive disorders (Steckhan et al., 2016). Furthermore, systemic leptin treatment has been found to lower immobility in rodents in FST and tail suspension tests, reflecting the antidepressant-like effect of leptin (Lu, Kim, Frazer & Zhang, 2006). Asakawa et al. (2003) also reported that leptin treatment could ameliorate anxiety-like behavior. In our study, we investigated anxiety and depressive-like behavior in *db/db* mice using FST, light-dark exploration, and open field tests. Increased immobility in FST, as well as a decrease in locomotor activity, have been previously reported in obese mice (Collin, Hakansson-Ovesjo, Misane, Ogren & Meister, 2000). During the open field test, *db/db* mice exhibited a decrease in basic movements compared with littermate WT controls. Similar changes in the locomotor behavior of *db/db* mice have been reported in previous studies (Hesse et al., 2010). Furthermore,



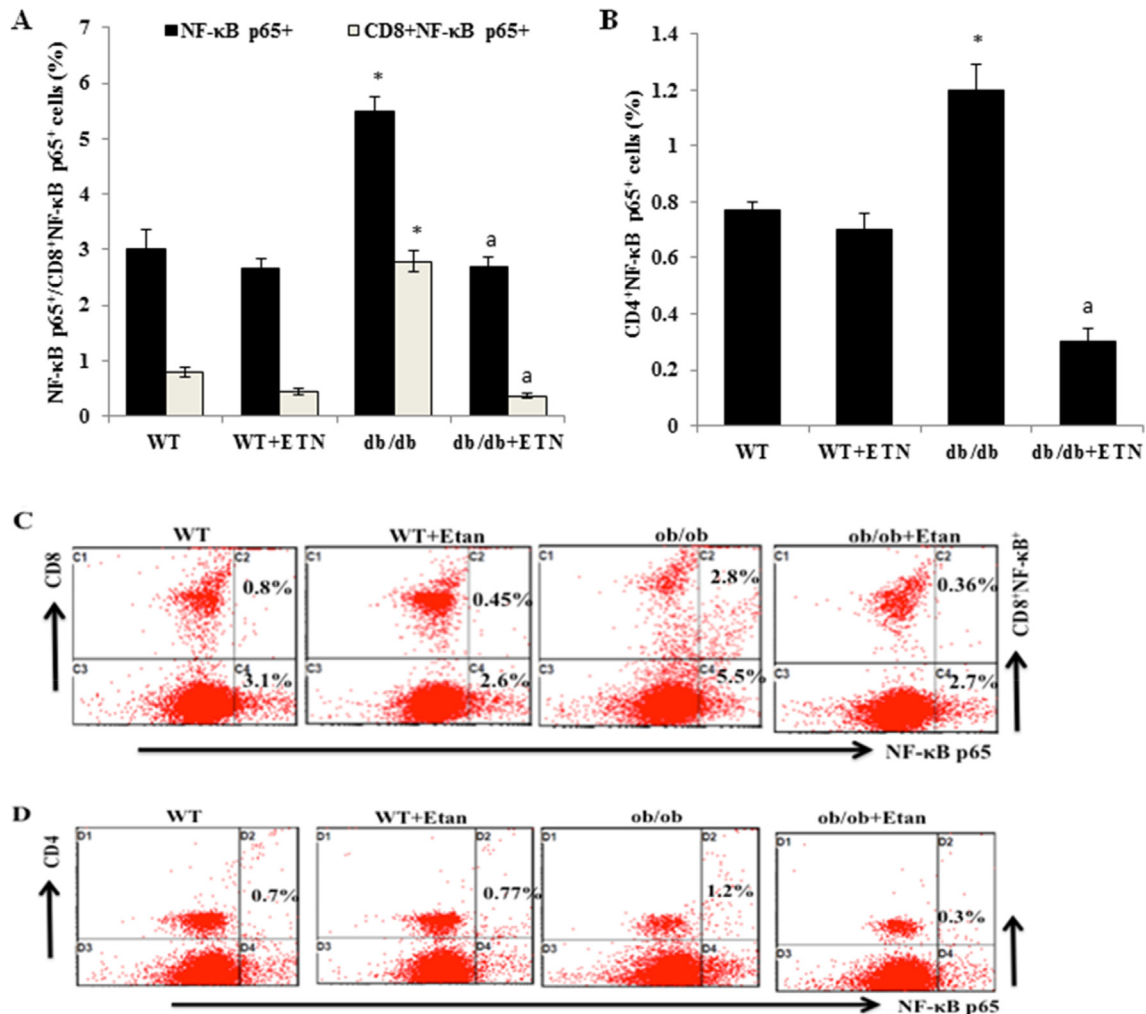
**Fig. 3.** Etanercept reversed the increase in IL-17A + and IL-17A-producing CD4 + and CD8 + T cells peripherally in *db/db* mice. **A** and **B**. The effects of ETN on IL-17A<sup>+</sup> and IL-17A<sup>-</sup> expressing CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the peripheral blood (analyzed using flow cytometry). **C**. Representative dot plots for one mouse from each group. \**P* < 0.05 compared with *db/db* saline-treated mice. Data are shown as mean ± SEM (*n* = 4).

hypo-locomotion activity has been observed in association with psychiatric disorders (Collin et al., 2000; Crawley et al., 1997). Previous studies have shown that *db/db* mice exhibit depressive and anxiety phenotypes (Asakawa et al., 2003; Diné et al., 2011; Guo & Lu, 2014; Sharma, Elased, Garrett & Lucot, 2010). In the present study there was no increase in immobility time in the FST among *db/db* mice; however, this could be because six animals were used which, in behavioral tests, may be a confounding factor.

Etanercept is a TNF- $\alpha$  inhibitor that, due to its molecular weight, does not cross the blood-brain barrier (Chang et al., 2017). Nevertheless, the peripheral administration of etanercept has been shown to reduce the symptoms of depression (Tyring et al., 2006). In line with this finding, studies have indicated that etanercept modulates neuronal inflammation (Kerfoot et al., 2006) and normalizes corticosterone induced synaptic changes (Brymer, Fenton, Kalynchuk & Caruncho, 2018). These central effects can be achieved indirectly by changing the level of circulating cytokines and inflammatory mediators. Research conducted by Campbell and his colleagues (2007) showed that the peripheral administration of etanercept significantly reduced neuronal inflammation in a rat model of brain trauma. They also identified

a correlation between these reductions and the hepatic synthesis of circulatory chemokines and neutrophil. In the current study, etanercept decreased immobility time in FST and reduced the time spent in corners in the open field test. These results suggest a reversal of depression/anxiety-like behavior in *db/db* mice as a result of administering etanercept.

Obesity is characterized by metabolic dysfunctions and is associated with peripheral as well as neuronal inflammation (Capuron, Lasselien & Castanon, 2017). Previous studies have shown that increased systemic or neuronal inflammation is linked to neurobehavioral deficits, including anxiety-related symptoms in chronic inflammatory conditions (Chen et al., 2015). Similarly, secreted pro-inflammatory cytokines induce neuroinflammation by impacting neurocircuitry, thereby leading to emotional/mood dysregulations (Miller, Haroon, Raison & Felger, 2013). Furthermore, the administration of anti-inflammatory agents appears to exert beneficial effects on depression and anxiety as evidenced by a reduction in anxiety-like behaviors in animal models of inflammatory diseases (Lee et al., 2018). Although the neuropsychological consequences of diabetes have been studied in considerable detail in clinical settings, there is a dearth of preclinical research identifying



**Fig. 4.** Etanercept reduced NF-κBp65 expressing CD4 + and CD8 + T cells peripherally in *db/db* mice. **A and B.** The effects of ETN on NF-κBp65<sup>+</sup> and NF-κBp65<sup>+</sup>-expressing CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the peripheral blood (analyzed using flow cytometry). **C.** Representative dot plots for one mouse from each group using flow-cytometric studies. \**P* < 0.05 compared with *db/db* saline-treated mice. Data are shown as mean ± SEM (*n* = 4).

correlations between neuropsychological consequences and diabetes.

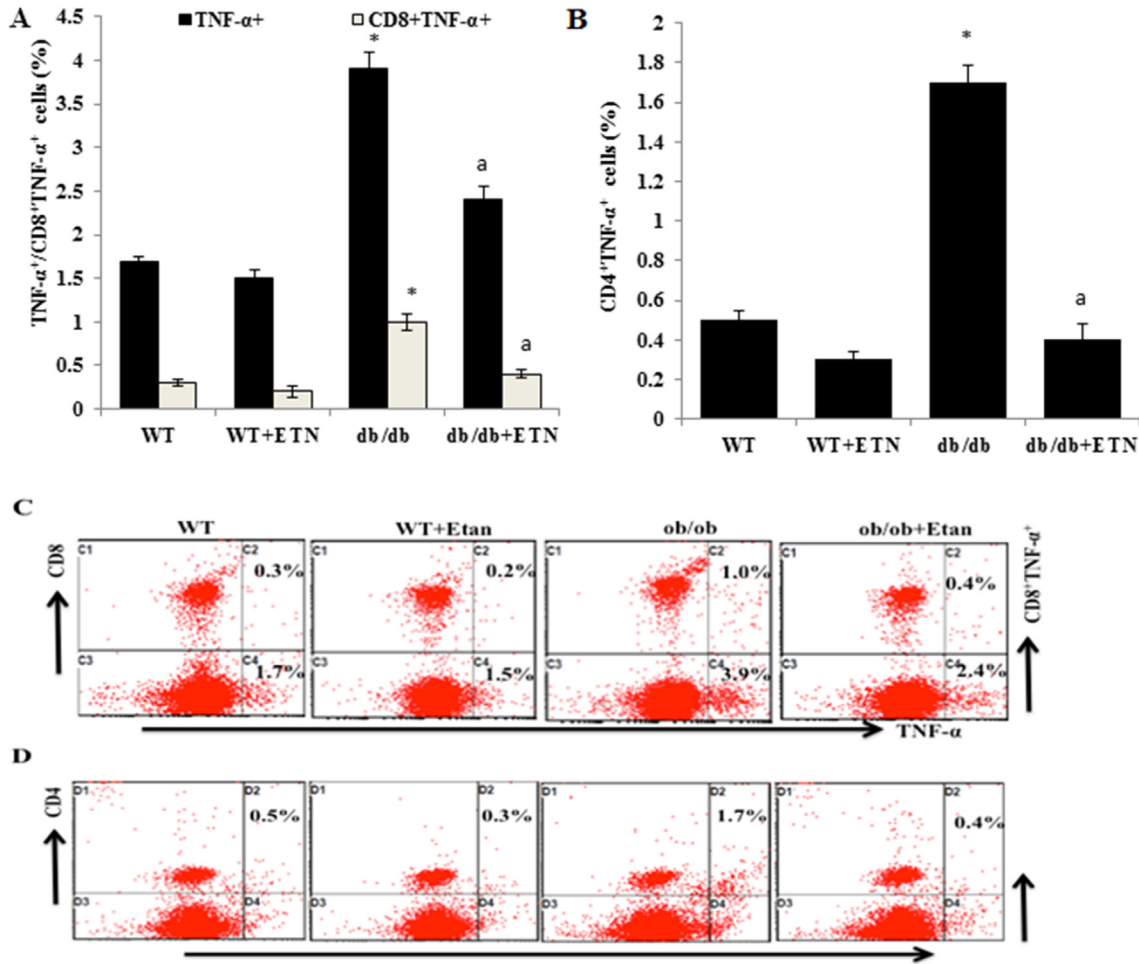
Previous studies have confirmed that stress/depression is associated with elevated systemic and neuronal inflammation, suggesting a crucial role for inflammation in the etiology of depression (Slavich & Irwin, 2014). IL-17A has also been found to play an essential role in neuroinflammatory diseases (Jadidi-Niaragh & Mirshafiey, 2011). Several studies have found that inflammation in the periphery could affect brain function, thereby causing the pathological changes associated with depression (Beurel et al., 2013; Waisman et al., 2015). Moreover, IL-17A has been found to be strongly associated with obesity-related systemic and adipose tissue inflammation (Chehimi, Vidal & Eljaafari, 2017). Therefore, IL-17A may be a common link between obesity-induced forms of depression. Based on these observations, we examined the effect of etanercept on IL-17A expression in *db/db* mice. We recorded a significant downregulation of IL-17A-producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells following etanercept administration. The results support our hypothesis that etanercept may be a beneficial therapy for the anxiety/depression symptoms associated with obesity.

We further observed that the *db/db* mice exhibited a substantial increase in TNF-α-producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the blood, and that this effect was reduced following etanercept treatment. TNF-α induces the development of anxiety-like behavior in normal

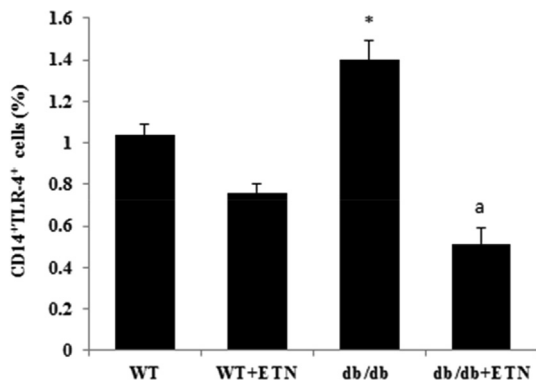
and obese mice (Andre et al., 2014; Jia et al., 2007; Takeuchi et al., 2006). In line with this finding, inhibition of TNF-α signaling by fluoxetine has been shown to improve depression-like symptoms (Todorovic & Filipovic, 2017). The TNF-α antagonist has also been found to reduce anxiety-like behavior in mice (Chen et al., 2013; Jia et al., 2007). Our results provide valuable evidence to suggest that a blockade of TNF-α signaling reduces obesity-associated inflammation as well as depression in *db/db* mice.

A previous study by Kassed and Herkenham (2004) showed that a deficiency in NF-κB signaling reduces anxiety-like behaviors in a mouse model. Another study by Bingham (2002) demonstrated that therapeutic targeting of NF-κB signaling reduces the production of pro-inflammatory mediators. In the current study, we found that NF-κB expression in CD4<sup>+</sup>/CD8<sup>+</sup> T cells significantly increases in *db/db* mice but can be downregulated by treatment with etanercept in the periphery. These results provide evidence to show that a blockade of TNF-α by etanercept may reduce systemic NF-κB activation. This might explain the potent anti-inflammatory effects of etanercept.

Recent data have reported that TLRs perform a critical function in learning and memory processes (Femenia et al., 2018). TLR-4 is involved in a multitude of physiological processes within the CNS that regulate stress/depression, including neuroinflammation and hippocampal neurogenesis (Liu, Buisman-Pijlman & Hutchinson,



**Fig. 5.** Etanercept reduced TNF-α<sup>+</sup> and TNF-α<sup>-</sup> expressing CD4<sup>+</sup> and CD8<sup>+</sup> T cells peripherally in *db/db* mice. **A and B.** The effects of ETN on TNF-α<sup>+</sup> and TNF-α<sup>-</sup> expressing CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the peripheral blood (analyzed using flow cytometry). **6C.** Representative dot plots for one mouse from each group. \**P* < 0.05 compared with *db/db* saline-treated mice. Data are shown as mean ± SEM (n = 4).



**Fig. 6.** The effects of ETN on TLR-4-producing CD14<sup>+</sup> cells in the peripheral blood (analyzed using flow cytometry). \**P* < 0.05 compared with *db/db* saline-treated mice. Data are shown as mean ± SEM (n = 4).

2014; Potter, Giedraitis, Johnson, Cox & Kohman, 2019). Previous studies have investigated the role played by TLR-4 in the regulation of anxiety-like behavior and sociability (Femenia et al., 2018). Another study by Shannonhouse et al. (2014) found that elevated-TLR-4 expression in the hypothalamus was associated with an increase in anxiety-like behavior and stress responses. These findings indicate that dysfunction in TLR-4 signaling may contribute to

systemic/neuronal inflammation as well as anxiety-like behavior in *db/db* mice. Therefore, etanercept-mediated reduction in TLR-4 signaling could be one of the factors that ameliorate anxiety/depression-like symptoms. Antidepressant treatment in patients with major depressive disorder has also been shown to normalize the expression of different TLRs, including TLR-4 (Hung, Huang, Kang, Huang & Huang, 2016).

**5. Conclusion**

The current study showed that increased systemic inflammation (as reflected by increased TNF-α/TLR-4 /NF-κB/IL-17A signaling) is associated with anxiety-like behavior in *db/db* mice. The blockade of TNF-α signaling by etanercept improved anxiety and depression-like behavior in *db/db* mice through the suppression of multiple inflammatory pathways. Therefore, our data suggest that a systemic blockade of TNF-α signaling by etanercept may be a promising approach to the treatment of obesity-associated anxiety/depression.

**Funding**

This research project was supported by a Research Initiative from the Prince Naif bin AbdulAziz Health Research Center, King Saud University Medical City.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgments

The authors acknowledge the help of the staff and use of the facilities of the Molecular and Cell Biology (MCB) Laboratory, a core research facility of the King Saud University (College of Dentistry) in collaboration with Prince Naif bin AbdulAziz Health Research Center.

## Author contributions

M.A.A., M.R.K., S.F.A., A. N., and T.K.A: contributed to the design of the work; the acquisition, analysis, and interpretation of the data; and drafted the manuscript. M.A.A. and M.R.K: prepared mouse samples and supervised and maintained the animal colony and animal genotyping in the laboratory. A.O.A: maintained the animal colony and animal genotyping in the laboratory. M. A. A., H. M. M., and M. R. K: contributed to behavioral experiments and behavioral data analysis. S.D.A: allowed behavioral experiments to be performed in his laboratory and provided intellectual support. S.F.A, A. N: Performed Flow cytometry. F.F.A., F. M. A., A. F. A., A.A: provided intellectual support and contributed to editing the manuscript.

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