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CD40-TNF activation in mice induces extended sickness behavior syndrome co-incident with but not dependent on activation of the kynurenine pathway

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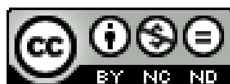

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CD40-TNF activation in mice induces extended sickness behavior syndrome co-incident with but not dependent on activation of the kynurenine pathway

Flurin Cathomas[a,b,1], Rene Fuertig[c,1], Hannes Sigrist[a], Gregory N. Newman[a], Vanessa Hoop[a,d], Manuela Bizzozzero[a], Andreas Mueller[e,f], Andreas Luippold[g], Angelo Ceci[c], Bastian Hengerer[c], Erich Seifritz[b,f], Adriano Fontana[e,f], Christopher R. Pryce[a,f,2]

[a] Preclinical Laboratory for Translational Research into Affective Disorders, Department of Psychiatry, Psychotherapy & Psychosomatics, Psychiatric Hospital, University of Zurich, Switzerland

[b] Department of Psychiatry, Psychotherapy & Psychosomatics, Psychiatric Hospital, University of Zurich, Switzerland

[c] CNS Diseases Research Germany, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach an der Riss, Germany

[d] ETH Zurich, Institute of Human Movement Sciences and Sport, Zurich, Switzerland

[e] Institute of Experimental Immunology, University of Zurich, Zurich, Switzerland

[f] Neuroscience Center, University and ETH Zurich, Switzerland

[g] Drug Discovery Support, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach an der Riss, Germany

[1] These authors contributed equally and share first authorship

[2] Corresponding author: Prof. Christopher Pryce, PLaTRAD, Department of Psychiatry, Psychotherapy & Psychosomatics, Psychiatric Hospital, University of Zurich, August Forel-Strasse 7, CH-8008 Zurich, Switzerland. Email: christopher.pryce@bli.uzh.ch, Tel +41 634 8921, Fax +41 634 8829

Abstract

The similarity between sickness behavior syndrome (SBS) in infection and autoimmune disorders and certain symptoms in major depressive disorder (MDD), and the high co-morbidity of autoimmune disorders and MDD, constitutes some of the major evidence for the immune-inflammation hypothesis of MDD. CD40 ligand-CD40 immune-activation is important in host response to infection and in development of autoimmunity. Mice given a single intra-peritoneal injection of CD40 agonist antibody (CD40AB) develop SBS for 2-3 days characterized by weight loss and increased sleep, effects that are dependent on the cytokine, tumor necrosis factor (TNF). Here we report that CD40AB also induces behavioral effects that extend beyond acute SBS and co-occur with but are not mediated by kynurenine pathway activation and recovery. CD40AB led to decreased saccharin drinking (days 1-7) and decreased Pavlovian fear conditioning (days 5-6), and was without effect on physical fatigue (day 5). These behavioral effects co-occurred with increased plasma and brain levels of kynurenine and its metabolites (days 1-7/8). Co-injection of TNF blocker etanercept with CD40AB prevented each of SBS, reduced saccharin drinking, and kynurenine pathway activation in plasma and brain. Repeated oral administration of a selective indoleamine 2,3-dioxygenase (IDO) inhibitor blocked activation of the kynurenine pathway but was without effect on SBS and saccharin drinking. This study provides novel evidence that CD40-TNF activation induces deficits in saccharin drinking and Pavlovian fear learning and activates the kynurenine pathway, and that CD40-TNF activation of the kynurenine pathway is not necessary for induction of the acute or extended SBS effects.

Highlights

- CD40 agonist antibody (CD40AB) induces acute sickness and, extending beyond this, decreased saccharin drinking and impaired classical fear conditioning
- CD40AB induces increased blood and brain activity of the kynurenine pathway co-incident with behavioral disruption
- CD40AB effects are mediated by the cytokine tumor necrosis factor, as demonstrated using etanercept
- A selective inhibitor of indoleamine 2,3-dioxygenase was effective in reversing CD40AB-induced kynurenine pathway activation but was without effect on acute and extended sickness

Key words: CD40; sickness behavior syndrome; depression; TNF; IDO; kynurenine pathway; reward motivation; fear learning; fatigue

1. Introduction

In infection and autoimmune disease, patients present with immune activation and inflammation in various organs. These responses are downstream of the host response in the case of infection, and effects of autoimmune T-cells and antibodies in the case of autoimmune disorders. CD40 is a member of the tumor necrosis factor receptor superfamily, and the CD40 ligand (CD40L, CD154)-CD40 immune-activation pathway is important in both the host response to infectious pathogens and development of autoimmunity (for review: Munroe, 2009; Peters et al., 2009). CD40L is expressed by activated $CD4^+$ T-cells, and binds to CD40 expressed on macrophages, dendritic cells, B-lymphocytes and endothelial cells (for review: Grewal and Flavell, 1998). As demonstrated in *in vitro* and animal studies, CD40 activation leads to cell migration and production of cytokines including tumor necrosis factor (TNF), interleukin- (IL-) IL-1β, IL-6, interferon- (IFN-) IFN-α, -β and -γ and IL-12 (for review: Chatzigeorgiou et al., 2009). Inflammation and hypertrophy of lymphoid tissues follow, characterized by loss of normal follicular structure and co-localization of activated T cells and B cells in lymphocytic clusters (Peters et al., 2009). Pronounced infiltrates of B cells, NK cells, $CD4^+$ T-cells, dendritic cells and macrophages occur in various organs, mainly the liver but also lung, pancreas and gastrointestinal tract (Kimura et al., 2006; Zhou et al., 2005). Furthermore, lymphadenopathy and splenomegaly occur due to myeloid cell hyperplasia and B-cell expansion. Whilst inhibition of CD40 or CD40L renders mice resistant to experimental autoimmune disorders, increased CD40L:CD40-induced immune-inflammation is associated with or directly involved in pathogenesis of human autoimmune disorders, including autoimmune hepatitis, primary biliary cirrhosis, inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis (for review: Peters et al., 2009). In addition to inflammation and organ dysfunction, patients with infection or autoimmunity also exhibit sickness behavior syndrome (SBS), including fatigue, malaise, decreased appetite, weight loss, reduced social activities, and cognitive impairment (for review: Dantzer et

al., 2008; Gold and Irwin, 2009). In mice, acute activation of CD40 signaling induces 2-3 day SBS in the form of weight loss, decreased motor activity and increased slow-wave sleep (Gast et al., 2013; Taraborrelli et al., 2011).

Major depressive disorder (MDD) is the most common psychiatric disorder and causes extensive individual morbidity and socioeconomic burden (Lopez et al., 2006). The core symptoms are low mood, reduced interest and pleasure, and fatigue, and additional symptoms include decreased appetite, weight loss, disturbed sleep and cognitive impairment (DSM-5, 2013; ICD-10, 1994). At the level of clinical phenotype therefore, there is large overlap between MDD and the SBS of infectious and autoimmune diseases (for review: Dantzer et al., 2008; Raison and Miller, 2013). Indeed, there is high comorbidity of MDD with autoimmune disorders, e.g. lifetime prevalence of 20-40% for MDD-rheumatoid arthritis (Bruce, 2008) and 50% for MDD-multiple sclerosis (Siegert and Abernethy, 2004). This high comorbidity is one of several lines of evidence consistent with an important role for immune-inflammation in the aetio-pathophysiology of MDD; another line is that chronic psychosocial stress, a major risk factor for MDD (Kendler et al., 2003; Kessler, 1997), also stimulates immune-inflammation (for review: Berk et al., 2013; Dantzer et al., 2008; Haroon et al., 2012; Miller et al., 2009; Rohleder, 2014).

Much of the evidence for the immune-inflammation basis of SBS and potentially MDD has been obtained using acute immune-inflammatory challenges in rodents (for review: Dantzer and Kelley, 2007; Kelley et al., 2003). Most widely used is an intraperitoneal (i.p.) injection of bacterial lipopolysaccharide (LPS), which stimulates toll-like receptor 4 and induces increased pro-inflammatory cytokine levels, including TNF, IFN-γ, IL-1β and IL-6, in the immune compartment, blood and brain (for review: Dantzer et al., 2008). The increased cytokine levels co-occur with an acute SBS that peaks 4-6 hours post-injection and subsides by about 24 hours, comprising features such as reduced food and water intake, decreased motor activity and increased slow-wave sleep

(for review: Dantzer et al., 2008). Behaviors with potential face validity for MDD symptoms (DSM-5, 2013; ICD-10, 1994) are also induced by immune-inflammation challenge, including decreased drinking of sweet-tasting fluid (gustatory reward), relevant to the core symptom of diminished interest-pleasure, and decreased Pavlovian/classical conditioning (learning and memory), relevant to the common symptom of cognitive impairment (Biesmans et al., 2013; Pugh et al., 1998; Thomson and Sutherland, 2005). These behavioral effects co-occur with and, for about 24 hours, extend beyond the classical SBS changes. Whether these latter behavioral effects should be considered as distinct from or rather as part of SBS ("extended SBS") is not just semantic but of theoretical and practical importance, and in particular in terms of whether they have different or shared aetio-pathophysiology. One shared candidate pathway for the mediation of both SBS and MDD-like behaviors is increased brain pro-inflammatory cytokine levels (for review: Boulanger, 2009; Capuron and Miller, 2011; Miller et al., 2009). A second candidate pathway is altered metabolism of tryptophan (Dantzer et al., 2008; Schwarcz et al., 2012): IFN-γ and TNF induce expression of indoleamine 2,3 dioxygenase (IDO) (O'Connor et al., 2009a), an intracellular enzyme expressed by dendritic cells, macrophages (periphery) and microglia (brain), that metabolizes tryptophan along the kynurenine (KYN) pathway (for review: Felger and Miller, 2012; Mellor and Munn, 2004; Schwarcz et al., 2012; Taguchi et al., 2014). In the mouse brain, IDO expression is increased at 24-48 hours after LPS treatment, coincident with LPS-induced behavioral effects (O'Connor et al., 2009b). Both tryptophan and KYN are transported readily across the blood-brain-barrier, and peripheral administration of KYN induces similar behavioral effects to those induced by LPS (O'Connor et al., 2009b). In periphery and brain, KYN catabolism results in increased levels of 3-OH-kynurenine (3-HK), quinolinic acid (QUIN) and/or kynurenic acid (KYNA). These increases can result in excitotoxicity and oxidative stress in various brain regions and cell types, mediated by

and impacting on various neurotransmitter systems, including glutamate, serotonin and dopamine (for review: Felger and Miller, 2012; Schwarcz et al., 2012).

For the translational study of the aetio-pathophysiology of SBS in autoimmune disorders, a mouse model of CD40-CD40L immune activation based on a monoclonal CD40 activating antibody (CD40AB) has been developed (Gast et al., 2013; Taraborrelli et al., 2011). As noted above, intraperitoneal (i.p.) injection of CD40AB induces a 2-3-day SBS in the form of weight loss and decreased activity associated with increased NREM sleep, low delta power and increased sleep fragmentation with increased brief awakenings (Gast et al., 2013). CD40AB-induced SBS is TNF-dependent, as evidenced by its absence in TNF receptor-1 knockout mice and its prevention in mice co-injected with the TNF blocker etanercept (Gast et al., 2013; Taraborrelli et al., 2011). The potential utility of this mouse model for the study of the relationship between immune-inflammation and MDD is evidenced by reports that MDD patients, compared to control subjects, have high blood levels of CD40 (Neubauer et al., 2013), soluble CD40L (Leo et al., 2006; Neubauer et al., 2013), TNF (Dowlati et al., 2010), IDO and KYN (Kim et al., 2012), and by the evidence that improved mood in MDD patients co-occurred with reduced blood levels of soluble CD40L (Leo et al., 2006; Selzhammer et al., 2013).

The first aim of the present study was to investigate the time course of effects of i.p. CD40AB on: SBS measures of food intake and body weight; SBS- and MDD-relevant behaviors of interest in gustatory reward, learning and memory in fear conditioning, and physical fatigue; peripheral and brain levels of pro-inflammatory cytokines, tryptophan, kynurenines and serotonin. The second aim was to investigate the effects of co-administration of the TNF blocker etanercept and CD40AB (both i.p.) to assess the importance of TNF in mediating the observed CD40AB effects on SBS, interest in reward and tryptophan catabolism. The third aim was to investigate the effects of repeated oral administration of an IDO inhibitor to block KYN pathway activation, on CD40AB

effects on SBS and interest in reward. As such, the study aimed to provide increased understanding of whether or not a causal pathway involving CD40, TNF and kynurenines contributed to the aetio-pathophysiology of SBS and/or MDD-relevant behavior. This would add important evidence with respect to the aetio-pathophysiology mediating between CD40 signaling, SBS and MDD in autoimmune disorders as well as in MDD *per se*.

2. Materials and methods

2.1. Animals

The study was conducted using adult male C57BL/6J mice aged 10-14 weeks (Janvier Labs, Le Genest-Saint-Isle, France). Unless otherwise stated, mice were maintained in littermate pairs. Cages were type 2L and contained sawdust and a sleeping igloo. Food (Complete pellet, Provimi, Kliba Ltd, Kaiseraugst, Switzerland) and water were both available continuously. Mice were maintained on a reversed 12:12 h light-dark cycle (lights off at 07:00 h) in an individually-ventilated cage system with temperature at 20-22 °C and humidity at 50-60 %. Naive mice were used for each experiment and were handled on each of three days prior to the experiment. *In vivo* experiments were conducted at the University of Zurich under license 170/2012 according to the regulations of the Veterinary Office, Canton Zurich, Switzerland, except one *in vivo* experiment (Section 2.8) which was conducted at Boehringer Ingelheim, Biberach, Germany under license 11-014 according to the regulations of the Regional Authority, Tuebingen, Germany.

2.2. CD40 agonist antibody

The stimulatory CD40 monoclonal antibody (CD40AB) was a rat IgG2a mouse-CD40 antibody (clone FGK4.5, Bio X Cell, West Lebanon, NH, USA). This was injected into the peritoneum (i.p.) at a dose of 10 mg/kg in PBS. As an antibody control, rat IgG2a isotope control (clone 2A3,

Bio X Cell) was injected i.p. at 10 mg/kg in PBS. Mice were injected once (day 0) at 18:45 h (Gast et al., 2013; Taraborrelli et al., 2011).

2.3. CD40AB effects on sickness measures

Naive mice were injected on day 0 with CD40AB (N=35) or IgG2a (N=18), and body weight and food consumption (weight of pellets eaten) were measured at 14:00 h daily. Five CD40AB and three IgG2a mice were sacrificed on each of days 2, 4, 5, 6, 7, 8 and 12, for blood and brain collection for determination of TNF and tryptophan metabolites (see Section 2.7) and also weighing of spleen.

2.4. CD40AB effects on saccharin consumption and preference

Home cage two-bottle test. Rodent reward sensitivity is typically studied using gustatory stimuli, with subjects drinking more of a sweet-tasting solution (e.g. saccharin, sucrose) than water in a two-bottle choice test, a preference that is reduced by chronic stress (Willner, 1997) and reduced for 1-2 days by LPS (Dantzer et al., 2008; Salazar et al., 2012). In the home-cage, naive mice were singly-housed and habituated to drinking out of 15 ml polypropylene centrifuge tubes (SuperClear 21008-214, VWR International) from which the tip had been removed. Two such tubes were attached adjacently to the cage lid each day at 08:00 h and removed at 16:00 h. Amount drunk per tube in ml was calculated as (tube weight (g) at 08:00 h - tube weight (g) at 16:00 h). On days -8 to -6, both tubes contained water to provide data on baseline daily water intake. On days -5 to 0, one tube contained water and the other 0.5% (W/V) saccharin (as sodium salt hydrate, S1002, Sigma) solution, with the left-right positioning of the two tubes alternated across days. This extensive saccharin pre-exposure was carried out to prevent conditioned taste aversion (CTA) of saccharin to CD40AB-induced sickness (Pacheco-Lopez and Bermudez-Rattoni, 2011). Mice were

injected with CD40AB (N=10) or IgG2a (N=10) at day 0. On days 1-10 following CD40AB/IgG2a injection, daily saccharin and water consumption were measured and also percent saccharin preference was calculated, as: (saccharin solution consumed (g)/(saccharin solution + water consumed)) x 100.

IntelliCage operant choice test. IntelliCage (NewBehavior, Zurich, Switzerland) is a system for automated continuous monitoring of activity and operant behavior of individual mice in their home-cage (Endo et al., 2011). Each IntelliCage, 55 (W) x 38 (L) x 20 (H) cm, was divided at the centre to give two independent cages (Cathomas et al., 2015). Four such divided IntelliCages were used with each placed in an attenuation chamber with a reversed 12:12 h dark-light cycle and ventilation. Naive mice were fitted with a subcutaneous transponder to record: visits to the operant devices located in each corner of the cage; operant nosepoke responses into a sensor (light-barrier) at the door in each corner that opened to allow access to a drinking bottle; and the number of licks, measured via electrical contact of the tongue with the drinking tip. Two siblings were placed separately in each cage-half. Mice were first habituated to the cage for 3 days with operant doors open, and then the doors were closed for 7 days so that mice had to make a nosepoke (operant response) to open a door and access the water bottles for 20 sec. This was followed by 6 days training with one water bottle and one saccharin solution bottle (0.5 %), the locations of which were alternated each day at 16:00 h. Mice were injected with CD40AB (N=8) or IgG2a (N=8) at day 0. On days 1 to 10, total visits, nosepokes and licks were scored in terms of frequency and time of occurrence.

2.5. Effects of CD40AB on locomotor activity, fear conditioning to tone and context, and unconditioned odor fear

Learning and memory of the association between neutral stimuli and an innately aversive stimulus can be studied using fear conditioning. This involves a neutral to-be-conditioned stimulus (CS e.g. auditory tone) or context (arena) being paired with an aversive unconditioned stimulus (US e.g. foot electroshock) and the CS and context thereby acquiring emotional significance similar to the US (LeDoux, 2012; Rescorla, 1988). One of the main responses elicited by the CS or context is freezing behavior. Both CS fear conditioning and freezing are dependent on the amygdala (Kim and Jung, 2006), whilst context fear conditioning is dependent on amygdala and hippocampus (Maren et al., 2013). Acute LPS challenge decreases conditioned fear expression for 1-2 days (Thomson and Sutherland, 2005); chronic psychosocial stress increases contextual and CS fear conditioning and expression (Azzinnari et al., 2014; Fuertig et al., in prep.).

Locomotor activity, fear conditioning, and freezing to an unconditioned fear odor were each measured using a Multi-Conditioning System (TSE Systems, Bad-Homburg, Germany; see (Azzinnari et al., 2014; Cathomas et al., 2015; Pryce et al., 2012). Briefly, a Plexiglas arena was placed on an electroshock grid floor, and a frame containing an infrared light-beam movement detection system surrounded the arena/grid. Four such units were each contained in an attenuating chamber with a ventilation fan, house lights (8 lux) and a loud speaker emitting low-level white noise. Prior to each session the arenas and the grids were wiped with 70% ethanol and the waste tray was rinsed and dried. Behavioral measures were locomotor activity in arbitrary units and duration of freezing episodes defined as no detection of any movement for a minimum of 2 sec.

Locomotor activity and CS fear conditioning. Naive mice were injected with CD40AB (N=12) or IgG2a (N=12) at day 0. On day 4, Locomotor activity and Habituation, mice were placed in the arena for 15 min without tone or footshock. Locomotor activity (distance moved in arbitrary units) and percent time freezing were measured using the automated system. On day 5, Conditioning,

following 120 sec adaptation, mice were exposed to 6 trials of a tone of 5 kHz and 85 dB presented for 20 sec with the final 2 sec overlapping with a 2 sec x 0.2 mA footshock. The inter-trial interval (ITI) was 120 sec. On day 6, Expression test, mice were placed in a modified context with a divider containing a door placed into the centre of the arena and a white instead of a black waste tray. Following 180 sec adaptation, mice were played 18 x 30-sec CS tone with 17 x 90-sec ITI. For Conditioning and Expression test, percent time spent freezing during tone and ITIs was scored.

Context fear conditioning. Naive mice were injected with CD40AB (N=12) or IgG2a (N=12) at day 0. On day 4, Conditioning, mice were placed in the arena and, following 180 sec adaptation, were exposed to 6 trials of 2 sec x 0.2 mA footshock with ITIs of 138 sec. On day 5, Expression test, mice were exposed to the same context for 18 x 60 sec trials. Percent time spent freezing per 60-sec trial was scored.

Unconditioned odor fear. Naive mice were injected with CD40AB (N=5) or IgG2a (N=5) at day 0. On day 5, Odor fear test, mice were placed in the arena, following 5-min habituation, a plastic dish (∅ = 4 cm x 1 cm) containing 1 ml of fresh, volatile 4.2 M trimethylamine solution (25% w/v in water, W324108, Sigma-Aldrich) was placed beneath the inactive grid floor. Trimethylamine is a chemosignal in mouse urine that is aversive at concentrations ≥ 1 M (Li et al., 2013). During 15 min, per cent time spent freezing per 60-sec block was scored.

2.6. Effects of CD40AB on treadmill running

In the CD40AB mouse model, sickness includes increased NREM sleep and decreased REM sleep (Gast et al., 2013). Whilst increased sleep is not a component of increased fatigue, it might be expected to correlate with it. To assess a fatigue-like state, mice were tested in terms of running on a treadmill (Panlab/Harvard Apparatus, Cornellà, Spain) inclined at 5° and with an electroshock grid at its lower end set at 0.20 mA (Azzinnari et al., 2014). Singly-caged, naive mice

were injected with CD40AB (N=5) or IgG2a (N=5) at day 0. On day 4 a training session was conducted and testing was conducted on day 5. The training session consisted of 2 min at a treadmill speed of 0 cm/sec, 5 min at 15-20 cm/sec at 1 min increments, and 5 min at 20 cm/sec. The test session consisted of 2 min at 0 cm/sec, 5 min at 20 cm/sec, and 20 min at 25 cm/sec i.e. test running speed. Measures, scored automatically, were the test duration at 25 cm/sec with a maximum duration of 20 min, and the total cumulative footshock duration with a maximum of 10 sec.

2.7. CD40AB effects on plasma and brain levels of TNF and tryptophan metabolites

In the same mice used to study sickness end points (Section 2.3), five CD40AB and three IgG2a mice were sacrificed on each of the days 2, 4, 5, 6, 7, 8 and 12 at 13:00-14:00 h by decapitation: trunk blood was collected into an EDTA-coated blood tube (Microvette 500, Sarstedt, Nümbrecht, Germany) and placed on ice. The brain was removed from the skull and frozen on powdered dry ice. Bloods were centrifuged at 3000 rpm x 4 °C x 10 min and plasma removed and stored in two aliquots per sample in cryotubes (LoBind, Vaudaux-Eppendorf). Plasma samples and brains were stored at -80°C prior to processing. The plasma level of TNF was measured using a multiplexed particle-based flow cytometric cytokine assay (Azzinnari et al., 2014; Marques-Vidal et al., 2011; Vignali, 2000). The lower limit of TNF detection was 0.5 pg/mL.

Frozen plasma and brain samples were transferred to Boehringer Ingelheim Biberach, Germany. Plasma levels of tryptophan (TRP), its kynurenine (KYN) pathway metabolites and serotonin (5-HT) were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS, full details to be published elsewhere and available on request). Plasma samples, calibration standards and quality controls were diluted with mobile phase A (0.2 % formic acid, 0.01 % trifluoror acetic acid, 1 % acetonitrile in water) and internal standard mix (d5-tryptophan,

d4-kynurenine, d3-quinolinic Acid, d5-kynurenic acid, d4-serotonin). Methanol was added for protein precipitation. Subsequently, samples were centrifuged and supernatant was evaporated to dryness under a gentle stream of nitrogen and reconstituted in mobile phase A. Brain levels of TRP, KYN metabolites and 5-HT were measured using LC-MS/MS. Frozen brains were sectioned coronally at 1.0 mm intervals using a stainless-steel brain matrix (Plastics One, model MMCS-1) and single-edge blades (Apollo Herkenrath, model 10-100-063) (Azzinnari et al., 2014). Ventral hippocampus, medial prefrontal cortex (infralimbic cortex and ventral prelimbic cortex) and dorsal raphe nucleus were microdissected from the sections using a brain punch (∅ = 1.00 mm, Stoelting Europe, model 57397) and a mouse brain atlas (Franklin and Paxinos, 2008): ventral hippocampus (2 biopsies/hemisphere) at bregma -2.8 to -3.9 mm; medial prefrontal cortex (1 biopsy/hemisphere) at bregma 2.1 to 1.2 mm; dorsal raphe nucleus (1 biopsy) at bregma -4.1 to -5.1 mm. All microdissection steps were conducted at -18 °C. Brain biopsies were stored at -80°C until analysis. A saline perfusion step was not included in the brain collection protocol: a pilot study in naive and LPS-treated mice demonstrated that tryptophan metabolite levels were comparable in brain tissue collected with and without saline perfusion; expression levels of haemoglobin genes (e.g. *Hba-a1*, *Hbb-a2*, also expressed by brain cells (Biagioli et al., 2009)) obtained in brain tissues collected without saline perfusion were markedly lower (<0.02%) than those measured in blood monocytes (Azzinnari et al., 2014; unpublished data). Brain biopsies and an internal standard mix (d5-tryptophan, d4-kynurenine, d3-quinolinic acid, d5-kynurenic acid, d4-serotonin) in 0.1% formic acid and ice-cold methanol were homogenized by sonication. After centrifugation the supernatant was evaporated to dryness under a gentle stream of nitrogen and reconstituted in mobile phase A. Calibration standards and quality control samples underwent the same procedure.

The LC-MS/MS system used to analyze plasma and brain samples consisted of a CTC HTC PAL Autosampler (CTC Analytics AG, Zwingen, Switzerland) and an Agilent 1200 Series liquid chromatography system (Micro Vacuum Degasser, Binary Pump SL, Thermostatted Column Compartment; Agilent Technologies, Waldbronn, Germany), coupled to an API 4000™ triple quadrupol mass spectrometer (AB Sciex, Darmstadt, Germany). Separation of analytes was achieved using a GRACE VisionHT C18 Basic column (3 µm, 100 x 2.1 mm; Grace Davison Discovery Sciences, Lokeren, Belgium) and a linear gradient elution profile. All standards, solvents and reagents used were of highest purity (LC-MS grade where available; Sigma Aldrich; CDN Isotopes, Quebec, Canada; Cambridge Isotope Laboratories Inc, Andover, USA; Buchem BV, Apeldoorn, Netherlands). Mobile phase A consisted of 0.2 % formic acid, 0.01 % trifluoracetic acid, 1 % acetonitrile, in water, and mobile phase B consisted of 0.2 % formic acid, 0.01 % trifluoracetic acid, 1 % water, in acetonitrile. To assess accuracy, quality control samples (from a pool of the study samples) were run at the end of every sample batch. Absolute analyte concentrations in calibration standards, samples and quality control samples were calculated based on the analyte signal in relation to the signal of the deuterated internal standards; the amount of internal standard added was selected with the aims of being in the range of endogenous analyte concentrations and attaining an adequate signal-to-noise ratio. As demonstrated in a pilot study: in plasma samples it was possible to conduct reliable measurement of TRP, KYN, 3-hydroxy-kynurenine (3-HK), kynurenic acid (KYNA), quinolinic acid (QUIN) and 5-HT; in small region-specific brain biopsies (~1 mg of tissue) it was possible to reliably measure TRP, KYN, 3-HK, and 5-HT, but KYNA or QUIN were, with the exception of dorsal striatum, below the lower limit of quantification (LLOQ), which was 0.25 nM and 5 nM, respectively. Absolute analyte concentrations were calculated as µmol/l or nmol/l for plasma and µmol/kg or nmol/kg tissue for brain regions. Full method validation and its application to other tissues/matrices will be described elsewhere.

2.8. CD40AB ± etanercept on saccharin drinking and levels of cytokines and tryptophan metabolites

To investigate whether TNF is essential in mediating CD40AB effects on saccharin drinking and tryptophan catabolism, etanercept, the extracellular ligand binding domain of the TNF2/p75 receptor, was used. In a first experiment a home cage 2-bottle test was conducted on days 1-6 post-treatment. Naive mice were allocated randomly to the four treatment groups Saline-IgG2a (SAL-IgG2a, N=6), Etanercept-IgG2a (ETAN-IgG2a, N=6), SAL-CD40AB (N=8) and ETAN-CD40AB (N=8). The same protocol was used as described above (Section 2.4) except that the saccharin concentration was reduced from 0.5% to 0.1%; this was done to reduce absolute saccharin consumption with the expectation of thereby increasing the sensitivity of the saccharin preference measure to CD40AB effects (and would also be expected to reduce any effect of CD40AB on sensitivity to the bitter taste of saccharin (Aubert and Dantzer, 2005)). Mice were injected i.p. with saline or etanercept (Enbrel, Amgen, 10 mg/kg) followed immediately by IgG2a or CD40AB, at 18:45 h on day 0.

A second experiment was conducted at Boehringer Ingelheim, Biberach, Germany. Naive mice were maintained under a 12/12 h light/dark cycle with light on at 06:00 h, with conditions otherwise similar to those in Zurich. Naive mice were allocated to four groups: SAL-IgG2a (N=6), ETAN-IgG2a (N=6), SAL-CD40AB (N=6) and ETAN-CD40AB (N=7). At 07:00 h, mice were injected i.p. with saline or etanercept followed immediately by IgG2a or CD40AB. At day 3 – the day at which the maximal reductions in saccharin preference and consumption were observed in the behavioral experiment – trunk blood was collected and hippocampus and dorsal striatum were dissected and snap frozen; plasma and brain samples were stored at -80°C prior to analysis of cytokines, TRP, kynurenine metabolites and 5-HT. For cytokine (TNF, IFN-γ, IL-1β, IL-6) determinations, hippocampal tissue was lysed in 10-fold volume of complete Tris lysis buffer by sonication, kept on

ice for 20-30 min and centrifuged at 14000 rpm for 10 min, and the supernatant was used for assay. Cytokines in plasma and hippocampal lysates were measured using Mouse V-Plex electrochemiluminescence kits for IFN-γ, TNF-α, IL-6 and IL1β (Meso Scale Discovery, Rockville, MD, USA) according to the manufacturer's protocol (assay sensitivity-lowest calibrator: IFN-γ 0.20 pg/ml, TNF 0.12 pg/ml, IL-6 1.0 pg/ml and IL1β 0.34 pg/ml). For TRP metabolites and monoamines, plasma samples were treated as above, and brain samples were homogenised in mobile phase A and subsequently treated as above; in dorsal striatum it was possible to reliably measure QUIN in CD40AB treated mice in addition to the other metabolites (lower limit of quantification (LLOQ) = 1 nM).

2.9. CD40AB ± IDO inhibitor on saccharin drinking and levels of cytokines and tryptophan metabolites

To inhibit Indoleamine-2,3-dioxygenase 1 (IDO1), a potent (both human and murine) and selective IDO1 inhibitor (hereafter IDOInh) was synthesized informed by previous studies that investigated IDO1 inhibition as a potential immunotherapeutic strategy for cancer treatment (see Liu et al., 2010; Yue et al., 2009). The *in vitro* effective concentration on murine IDO1 to achieve a 50% reduction in KYN synthesis (EC_{50}) in a cellular assay was 390 nM. Selectivity for IDO1 relative to tryptophan 2, 3-dioxygenase (TDO) (EC_{50} TDO: EC_{50} IDO1) was 180. These efficacy and selectivity estimates for IDOInh are higher than those reported for 1-methyltryptophan (Hou et al., 2007; Lob et al., 2014), the IDO inhibitor used to-date to study effects of KYN-pathway inhibition (Dobos et al., 2012; Kiank et al., 2010; O'Connor et al., 2009b). Using a single IDOInh dose of 200 mg/kg p.o., the time required to reach peak plasma exposure was 1.3 h, and the mean residence time (average time that an IDOInh molecule remains in the blood) was 4.6 h. Plasma protein-unbound exposure at 2 h was 680 nM, about 1.8x *in vitro* EC_{50}. Cerebrospinal fluid exposure at 2 h was 60

nM, about 0.2x *in vitro* EC_{50}. After induction of high plasma kynurenine by 1 mg/kg LPS 24 h previously, the *in vivo* EC_{50} in plasma at 1 h post-administration was 10-30 mg/kg p.o. The IDOInh dosing regimen selected for this experiment was 75 mg/kg p.o. twice per day.

Naive mice were allocated randomly to four treatment groups: IgG2a-Vehicle (IgG2a-VEH, N=7), IgG2a-IDO inhibitor (IgG2a-IDOInh, N=8), CD40AB-VEH (N=7) and CD40AB-IDOInh (N=8). Mice were injected i.p. with CD40AB or IgG2a at 18:45 h on day 0. A home cage 2-bottle test (0.1 % saccharin) was conducted on days 1-5. Beginning on day 1, mice were administered p.o. with IDOInh (75 mg/kg/8 ml) or VEH (0.5% Natrosol in water) twice per day at 08:00 and 16:00 h on days 1-5. At 2 h after the final administration on day 5, mice were decapitated, and trunk blood and brain samples collected and processed as described above, with brain analysis conducted using ventral hippocampus (vHIPP).

2.10. Statistical analysis

Statistical analysis was performed with SPSS (version 20, SPSS Inc., Chicago IL, USA) and Graph Pad Prism (6.04). Analysis of variance (ANOVA) was conducted with a between-subject factor of Treatment e.g. (IgG2a, CD40AB), (SAL-IgG2a, ETAN-IgG2a, SAL-CD40AB, ETAN-CD40AB) and, depending on experiment, a within-subject factor of Day. *Post hoc* testing was conducted using the Bonferroni test or Tukey’s test. Statistical significance was set at $p<0.05$. Where an estimate of variance is given this is the standard deviation (SD).

3. Results

3.1. CD40AB induced sickness

For each sickness end point there was no day effect in IgG2a mice and data from all days were pooled. There was a Group (i.e. IgG2A days 2-12, CD40AB day 2, CD40AB day 4, etc.) effect

on food consumption ($F(7,43)=8.09$, $p<0.0005$; Fig. S1A): compared to IgG2a mice, CD40AB mice consumed less food at day 2 specifically. There was a Group effect on body weight ($F(7,44)=37.25$, $p<0.0005$; Fig. S1B): compared to IgG2a mice, CD40AB mice exhibited reduced body weight at days 2, 4 and 5. They also exhibited day-on-day recovery at days 2-4 ($p<0.05$) and 4-5 ($p<0.02$). There was a Group effect on spleen weight ($F(7,44)=52.80$, $p<0.0005$; Fig. S1C); CD40AB mice exhibited splenomegaly at days 2-8. For each experiment reported below, body weight was decreased in CD40AB versus IgG2a mice at days 1-4 or 1-5 ($p<0.0005$), demonstrating CD40AB efficacy.

3.2. CD40AB decreased saccharin preference, consumption and operant responding

Home cage two-bottle test. In the home-cage, mice presented with two water bottles drank 3.97±0.52 g per day (mean±SD, days -8 to -6, Fig. 1A). Daily baseline saccharin solution consumed was 5.95±1.50 g (days -2 to 0, Fig. 1B); therefore, mice exhibited increased daily fluid consumption when saccharin solution was available (paired $t_{(38)}=5.07$, $p<0.0005$). Mice exhibited a marked saccharin preference and were allocated to IgG2a (85±8 %) or CD40AB (85±6 %) by counterbalancing on this measure (days -2 to 0, Fig. 1C). Following CD40AB/IgG2a injection (day 0), for saccharin preference there was a Treatment x Day (0 to 10) interaction ($F(15,270)=4.44$, $p<0.001$; Fig. 1C): saccharin preference in CD40AB mice was lower than in IgG2a mice on days 1-3. For absolute saccharin consumption there was also a Treatment x Day interaction ($F(15,270)=12.16$, $p<0.001$; Fig. 1B): CD40AB mice drank less saccharin than did IgG2a mice on days 1-7. Compared to baseline water consumption (ca. 4 g/day, Fig. 1A), CD40AB mice exhibited reduced total fluid intake on days 1-4 and from day 5 baseline fluid intake was restored (Fig. 1B). For absolute water consumption, there was no effect involving Treatment ($p\geq0.46$; Fig. 1A).

FIGURE 1 ABOUT HERE

IntelliCage operant choice test. In a new cohort in IntelliCages, mice had stable baseline levels (days -5 to 0) for visiting the saccharin corner (Fig. 1D), nosepoking for saccharin (Fig. 1E), and licking/drinking saccharin (Fig. 1F); the same was true for water (data not shown). At baseline there was only a marginal preference to visit (Fig. S2A) and nosepoke (Fig. S2B) at the saccharin corner, which was probably due to daily alternation of saccharin and water bottles and environmental monitoring by the mice; there was a marked baseline saccharin preference in terms of licks, 89±14 % and 88±16 %, respectively, in mice allocated subsequently to IgG2a and CD40AB groups (Fig. S2C). CD40AB effects were as follows for days 0 to 10: For absolute number of visits to the saccharin corner there was a Treatment x Day interaction ($F(15,210)=6.07$, $p<0.001$; Fig. 1D): CD40AB mice made less visits than IgG2a mice on days 1-3. For % visits to the saccharin corner there was no effect of CD40AB ($p \geq 0.27$; Fig. S2A). For absolute number of nosepokes at the saccharin corner there was a Treatment x Day interaction ($F(15,210)=4.01$, $p<0.001$; Fig. 1E): CD40AB mice made less nosepokes than IgG2a mice on days 1-2 and 4-7. For % nosepokes at the saccharin corner there was no effect of CD40AB ($p \geq 0.58$; Fig. S2B). For absolute number of licks at the saccharin bottle there was a Treatment x Day interaction ($F(15,210)=2.63$, $p<0.001$; Fig. 1F): CD40AB mice made less licks than IgG2a mice on days 1-2. For % licks to the saccharin bottle there was no effect of CD40AB ($p \geq 0.57$; Fig. S2C).

3.3. CD40AB impaired classical conditioning without affecting locomotor activity or US fear

To assess CD40AB effects on locomotor activity, on day 4 after CD40AB/IgG2a, mice were placed in the conditioning arena without CS or US. There was no effect of CD40AB on locomotor activity ($p=0.09$; Fig. 2A). Furthermore, IgG2a and CD40AB mice each spent a low and similar % time freezing ($p=0.14$; Fig. 2B). On day 5 mice were exposed to six CS-US trials (Conditioning).

There was a main effect of CS-US Trial ($F(2,44)=40.29$, $p<0.001$; Fig. 2C) indicating incremental acquisition of fear freezing to the CS. There was also a Treatment x Trial interaction ($F(2,44)=3.7$, $p<0.04$): compared to IgG2a mice, CD40AB mice exhibited decreased acquisition of freezing in trials 3-4 ($p<0.01$) and 5-6 ($p<0.03$). There was no effect of Treatment on locomotor reactivity to the US (arbitrary units/sec): IgG2a 847±167 a.u./sec, CD40AB 745±226 a.u./sec ($p=0.13$), suggesting that CD40AB did not affect pain sensitivity. On day 6 mice were placed in a modified context and exposed to 18 CS trials without US (Expression test). There were main effects of Treatment ($F(1,22)=16.29$, $p<0.001$; Fig. 2D) and CS Trial ($F(5,110)=15.6$, $p<0.001$): CD40AB mice expressed less freezing than IgG2a mice across CS trials, and both groups exhibited a similar rate of decrease in fear expression across CS trials.

In a new mouse cohort, exposure to the US in the absence of a CS allowed for the study of contextual fear conditioning. On day 4 after CD40AB/IgG2a, mice were exposed to six US trials (Conditioning). There was a main effect of Interval ($F(2,44)=49.45$, $p<0.001$; Fig. 2E) indicating incremental acquisition of fear freezing to context. There was no effect of Treatment, neither as a main effect ($p=0.08$) nor in interaction with Interval ($p=0.11$). On day 5 mice were exposed to the context for 18 x 1-min intervals (Expression test). There was a main effect of Interval ($F(5,110)=10.64$, $p<0.001$) indicating decreased freezing across intervals, and no effect of Treatment ($p\geq0.14$) (Fig. 2F).

In a new mouse cohort, to study whether the reduced freezing induced by CD40AB observed in the CS-US paradigm was due to impaired learning-memory or rather a decrease in US fear *per se*, CD40AB effect on reactivity to an aversive odor-US to which rodents exhibit spontaneous freezing behavior was studied (Li et al., 2013). On day 5 after CD40AB/IgG2a, during arena habituation, IgG2a (0.3±0.4 %) and CD40AB (1.1±1.7 %) mice spent a low and similar % time freezing ($p=0.38$; Fig. 2G). Following introduction of the odor there was a main effect of Time

($F(4,32)=16.04$, $p<0.001$), indicating sustained development of freezing, and no effect of Treatment ($p\geq0.79$) (Fig. 2G). The absence of a CD40AB effect on freezing to the odor US supports the interpretation that CD40AB impairs CS-US learning-memory.

FIGURE 2 ABOUT HERE

3.4. No effect of CD40AB on treadmill running

In a new mouse cohort, after CD40AB/IgG2a on day 0, a treadmill habituation session and treadmill test were conducted on days 4 and 5, respectively. There was no CD40AB effect on total duration of footshocks received at habituation (data not shown) or test: IgG2a mice 4.8±3.2 sec, CD40AB mice 4.0±3.2 sec (*t*-test, $p=0.68$). Maximum test duration was 1200 sec, and the total running times achieved by IgG2a and CD40AB mice were 1174±63 and 1112±215 sec, respectively ($p=0.51$).

3.5. CD40AB activated blood TNF and KYN pathway in blood and brain

In plasma and brain-tissue samples obtained from the same cohort of mice that provided data on sickness end points (Fig. S1), we investigated CD40AB effects on TNF, kynurenines (Fig. 3B) and 5-HT. For plasma TNF there was a Group effect ($F(7,35)=28.87$, $p<0.0005$; Fig. 3A) with CD40AB mice exhibiting increased plasma TNF levels at days 2-5 relative to IgG2a mice, as expected (Gast et al., 2013; Taraborrelli et al., 2011). For plasma TRP there was no effect of Group ($p=0.13$; Fig. 3C). There were Group effects, indicating increased plasma levels in CD40AB relative to IgG2a mice on one or more days, for: KYN ($F(7,36)=47.97$, $p<0.0005$; Fig. 3D), 3-HK ($F(7,36)=35.44$, $p<0.0001$; Fig. 3E), and QUIN ($F(7,36)=24.1$, $p<0.0001$; Fig. 3F). There was no Group

effect for KYNA ($p=0.15$; Fig. 3G). CD40AB led to a significant decrease in plasma 5-HT ($F(7,36)=5.06$, $p<0.0005$; Fig. 3H).

FIGURE 3 ABOUT HERE

In brain tissue, CD40AB effects on TRP, KYN, 3-HK and 5-HT were measured in vHIPP (Fig. 4, Table S1), medial prefrontal cortex (mPFC, Table S1), and dorsal raphe nucleus (DRN, Table S1). In vHIPP there were Group effects, indicating increased levels in CD40AB versus IgG2a mice, on several days, for TRP ($F(7,32)=5.81$, $p<0.0002$; Fig. 4A), KYN ($F(7,32)=37.72$, $p<0.0001$; Fig. 4B) and 3-HK ($F(7,32)=46.79$, $p<0.0001$; Fig. 4C); there was no Group effect for 5-HT ($p=0.09$; Fig. 4D). In mPFC (Table S1), there was a Group effect for TRP ($F(7,31)=4.34$, $p<0.002$) but no significant post hoc effect; CD40AB induced increases for several days in KYN ($F(7,31)=17.80$, $p<0.0001$) and 3-HK ($F(7,30)=22.66$, $p<0.0005$), and was without effect on 5-HT ($p=0.24$). In DRN (Table S1), there were CD40AB-induced increases on one or more days for TRP ($F(7,30)=3.814$, $p<0.005$), KYN ($F(7,30)=26.92$, $p<0.0005$) and 3-HK ($F(7,27)=10.06$, $p<0.0005$), and no effect on 5-HT ($p=0.58$).

FIGURE 4 ABOUT HERE

3.6. Etanercept blocked CD40AB effects on sickness, saccharin drinking and KYN pathway

To investigate causal involvement of TNF in CD40AB-induced sickness and decreased saccharin drinking, experiments were conducted using co-administration of the TNF blocker etanercept (ETAN) with CD40AB. Firstly, effects on sickness and the home cage two-bottle test were investigated (Fig. 5). For body weight there was an interaction of Treatment x Day ($F(21, 168)=13.76$, $p<0.0005$; Fig. 5A): whereas SAL-CD40AB mice lost body weight as expected there was

no effect of ETAN-CD40AB relative to the two control groups. For saccharin preference there was a Treatment x Day interaction ($F(21, 168)=4.53$, $p<0.0005$; Fig. 5B): SAL-CD40AB mice exhibited reduced saccharin preference on days 1-3/4 relative to the other three groups, whilst ETAN-CD40AB mice maintained a high preference equivalent to that of the control groups. For absolute saccharin consumption there was a Treatment x Day interaction ($F(21, 168)=7.99$, $p<0.0005$; Fig. 5C): SAL-CD40AB mice exhibited reduced saccharin consumption on days 1-3/4 relative to the other three groups, whilst ETAN-CD40AB mice maintained high consumption. For absolute water consumption there was a Treatment x Day interaction effect ($F(21, 168)=2.48$, $p<0.001$; Fig. 5D) due to minor spontaneous differences at day 0 only. For spleen weight there was a Treatment effect ($F(3, 24)=143.53$, $p<0.0005$; Fig. S3A): whereas both SAL-CD40AB and ETAN-CD40AB mice exhibited splenomegaly relative to control groups, ETAN-CD40AB mice exhibited decreased splenomegaly relative to SAL-CD40AB.

FIGURE 5 ABOUT HERE

In a second experiment, naive mice were sacrificed at day 3 after treatment, and TNF, IFN-γ, IL-6, IL-1β, TRP and kynurenines were measured in blood (Fig. S4, Table S2) and brain. For plasma TNF, there was a Treatment effect ($F(3, 19)=15.69$, $p<0.0001$; Fig. S4A); both SAL-CD40AB and ETAN-CD40AB mice exhibited increased levels relative to control groups (with the high TNF levels in ETAN-CD40AB mice and typical TNF levels in ETAN-IgG2a mice expected to be biologically inactive). There was a Treatment effect for plasma IFN-γ ($F(3, 23)=24.09$, $p<0.0001$; Fig. S4B); SAL-CD40AB mice exhibited increased levels relative to each of the other groups. Plasma IL-6 was markedly elevated in some ETAN-IgG2a mice specifically ($F(3, 19)=5.01$, $p<0.01$; Table S2), and there were no effects of ETAN or CD40AB on IL-1β (Table S2). There was a Treatment effect for

plasma TRP ($F(3, 19)=9.34$, $p<0.0005$; Fig. S4C): both CD40AB groups exhibited decreased TRP relative to one or both control groups. There were Treatment effects for plasma KYN ($F(3, 19)=190.80$, $p<0.0001$; Fig. S4D), plasma 3-HK ($F(3, 19)=19.45$, $p<0.0001$; Fig. S4E) and plasma QUIN ($F(3, 19)=76.36$, $p<0.0001$; S4F): as expected, SAL-CD40AB mice exhibited increases in these plasma kynurenines relative to control mice, and they were lower in ETAN-CD40AB versus SAL-CD40AB mice, with levels in the former similar to those in control mice. There was a Treatment effect for plasma KYNA ($F(3, 19)=7.52$, $p<0.002$; Table S2), with levels similarly reduced in ETAN-IgG2a, SAL-CD40AB and ETAN-CD40AB mice relative to SAL-IgG2a mice. There was a Treatment effect for plasma 5-HT ($F(3, 19)=7.12$, $p<0.003$; Table S2); SAL-CD40AB mice exhibited decreased 5-HT relative to control mice, and this was also the case for ETAN-CD40AB mice.

In brain tissues from the same subjects (Fig. 6, Fig. S5, Table S3), for vHIPP there was a Treatment effect for TNF ($F(3, 19)=3.09$, $p=0.05$; Fig. S5A) with ETAN-CD40AB mice exhibiting increased TNF versus SAL-IgG2a mice, and for IFN-γ ($F(3, 19)=3.79$, $p<0.02$; Fig. S5B) with SAL-CD40AB mice exhibiting increased IFN-γ versus control mice. There were no Treatment effects for IL-6 or IL-1β (Table S3). There was no Treatment effect for TRP ($p=0.47$; Fig. S5C). There were Treatment effects for KYN ($F(3, 19)=69.14$; $p<0.0001$; Fig. S5D) and 3-HK ($F(3, 19)=43.37$, $p<0.0001$; Fig. S5E): SAL-CD40AB mice exhibited marked increases relative to control mice and ETAN-CD40AB mice. There were no Treatment effects for 5-HT (Table S3). In dorsal striatum (Fig. 6, Table S3) there was no Treatment effect for TRP ($p=0.51$; Fig. 6A). There were Treatment effects, due to increased levels in SAL-CD40AB mice relative to each of the other three groups, for KYN ($F(3, 19)=55.85$, $p<0.0001$; Fig. 6B) and 3-HK ($F(3, 19)=102.50$, $p<0.0001$; Fig. 6C). In dorsal striatum specifically it was also possible to measure QUIN reliably in CD40AB treated mice: there was a Treatment effect ($F(3, 17)=7.70$, $p<0.004$; Fig. 6D) with levels at or below assay LLOQ for control

and ETAN-CD40AB mice and detectable and therefore relatively high in SAL-CD40AB mice relative to each of these groups. There were no Treatment effects for striatal 5-HT (Table S3).

FIGURE 6 ABOUT HERE

3.7. IDO inhibition was without effect on CD40AB-induced sickness and saccharin drinking despite blocking activation of KYN pathway

To investigate causal involvement of the activated KYN pathway downstream of TNF in CD40AB-induced sickness and decreased saccharin drinking, an experiment was conducted using twice per day oral administration of an IDO inhibitor. For body weight there was an interaction of Treatment x Day ($F(15, 130)=16.61, p<0.001$; Fig. 7A): both CD40AB-VEH and CD40AB-IDOInh mice exhibited weight loss relative to control groups (IgG2a-VEH, IgG2a-IDOInh). In the home cage two-bottle test, for saccharin consumption there was a Treatment x Day interaction ($F(15,130)=5.43, p<0.001$; Fig. 7B): both CD40AB-VEH and CD40AB-IDOInh mice displayed reduced saccharin consumption on days 1-2 compared to control groups. For spleen weight there was a Treatment effect ($F(3,26)=118.32$, $p<0.001$; Fig. S3B): both CD40AB-VEH and CD40AB-IDOInh mice showed splenomegaly relative to control groups, and CD40AB-IDOInh mice exhibited increased splenomegaly relative to CD40AB-VEH mice.

In plasma samples (Fig. 7, Table S4), there were Treatment effects for KYN ($F(3, 24)=237.3$, $p<0.0001$; Fig. 7C), 3-HK ($F(3, 24)=48.80$, $p<0.0001$; Table S4) and QUIN ($F(3, 24)=21.03$, $p<0.0001$; Table S4). As expected, CD40AB-VEH mice exhibited increases in plasma levels of these kynurenines compared to IgG2a-VEH mice, and the CD40AB effects were blocked in CD40AB-IDOInh mice relative to CD40AB-VEH mice (also, IgG2a-IDOInh mice exhibited decreases in plasma kynurenines relative to IgG2a-VEH mice). There was also a Treatment effect for KYNA (F(3,

24)=4.25, p<0.02; Table 4), with CD40AB-IDOInh mice exhibiting reduced levels relative to the other groups. For vHIPP (Fig. 7, Table S4), there was a Treatment effect for KYN (F(3, 24)=21.53, p<0.0001; Fig. 7D), with the expected observed increase in CD40AB-VEH mice being blocked in CD40AB-IDOInh mice. There was a Treatment effect for TRP (F(3, 24)=4.67, p<0.02), with CD40AB-IDOInh mice exhibiting increased TRP compared to control groups. No effects were observed for 5-HT (Table S4).

4. Discussion

In mice, CD40 agonist antibody caused decreased consumption of and motivation for saccharin and decreased CS fear learning, and these effects were maintained beyond the period of SBS defined by less food consumption and loss of body weight. The sustained behavioral effects were preceded by increased blood TNF and blood and brain IFN-γ, and co-occurred with increased kynurenines in blood and brain. Co-administration of etanercept with CD40AB prevented each of sickness, decreased saccharin drinking, increased blood IFN-γ, and increased blood-brain kynurenines. Repeated oral IDO1 inhibitor administration was also effective in preventing CD40AB-induced increased blood and brain kynurenines; however, it was without effect on sickness and decreased saccharin drinking. Therefore, whilst increased kynurenine levels have been demonstrated to underlie behavioral effects induced by LPS and have been proposed to contribute to MDD, they are not necessary for CD40-TNF-induced sickness and decreased saccharin drinking.

4.1. A continuum of sickness, decreased saccharin drinking and impaired fear learning effects of CD40AB

Previous studies have described that CD40AB induces sickness as weight loss, decreased motor activity and increased slow-wave sleep, measured at days 1-2 (Gast et al., 2013; Taraborrelli et al., 2011). In the present study, weight loss co-occurred with decreased food consumption on day 2, and by days 3-4 mice had resumed normal eating and were increasing in body weight. With respect to saccharin drinking, non-manipulated mice exhibited increased absolute fluid consumption with saccharin solution versus water only, and had 90% preference for saccharin over water. The absolute increase in fluid consumption beyond baseline drinking constitutes "reward-drinking", i.e. high motivation for the taste of saccharin, as does the high preference for saccharin over water. In standard cages and with the two-bottle test, CD40AB induced decreased saccharin preference at days 1-3, a time period coincident with the sickness markers of decreased feeding and weight loss. CD40AB induced decreased absolute saccharin consumption at days 1-7: during days 1-4 mice consumed less total fluid than at baseline, and at days 5-7 they exhibited baseline drinking without additional reward-drinking. One potential explanation of why saccharin preference recovered prior to absolute saccharin consumption is that the sweet taste of saccharin likely predicts high calorific content and the earlier recovery of saccharin preference could reflect the high energy need of mice after weight-loss sickness, whereas saccharin consumption is possibly a more robust measure of non-essential reward motivation under these conditions. Another potential explanation is that CD40AB led to increased sensitivity to the bitter component of the overall taste of saccharin, and that this sickness-related effect led to the persistent decreased absolute consumption. Such an effect has been reported for LPS when saccharin solution is presented at the concentrations used in the present study (Aubert and Dantzer, 2005). In IntelliCages, the corner locations of saccharin and water were switched daily and mice distributed their visits and nosepokes almost equally between corners; nonetheless, their saccharin preference in terms of drinking (licks) was 80-90% and therefore similar to that observed

in standard cages. CD40AB decreased IntelliCage saccharin and water drinking at days 1-2, visits at days 1-3 and nosepokes at days 1-7. Therefore, the duration of the CD40AB effect on nosepoking was the same as that on absolute saccharin drinking in the standard-cage two bottle test. Visits and nosepokes are goal-directed operant responses, and these occurred less frequently after CD40AB such that mice concentrated their drinking into fewer operant episodes. Taken together, the two experiments provide evidence for a 7-day period of decreased motivation for saccharin drinking induced by CD40AB, with decreased reward motivation and increased bitter-taste sensitivity both likely to contribute to this state. Using the two-bottle test, LPS has been demonstrated to induce a 1-2 day decrease in preference for and/or absolute consumption of sucrose or saccharin solution (Aubert and Dantzer, 2005; Biesmans et al., 2013; Salazar et al., 2012; personal observation).

At day 4 there was no effect of CD40AB on locomotor activity in a novel environment. This suggests that the decreased activity observed in the home cage that is a major component of SBS at days 1-2 (Gast et al., 2013; Taraborrelli et al., 2011) has subsided by day 4. It clearly indicates that the subsequent fear conditioning experiment was not confounded by CD40AB effects on baseline locomotion or freezing levels. The effects of CD40AB on fear learning-memory were investigated at days 4-6 and therefore after sickness (weight-loss) recovery. CD40AB induced decreased learning of tone-footshock association. This effect was manifested as a decrease in freezing acquisition and also as a proportionate decrease in next-day fear expression to the CS. That the expression deficit was proportional to the learning deficit indicates that the CD40AB effect was specific to learning and did not affect memory consolidation-retrieval. Synaptic plasticity in the amygdala is critical for CS-US fear learning (Ehrlich et al., 2009; Kim and Jung, 2006). CD40AB did not affect context-US fear learning-memory, where synaptic plasticity in amygdala and particularly hippocampus is critical (Maren et al., 2013). Studies of LPS effects on

conditioning have used a different design to that used here: the focus has been on contextual rather than CS fear learning-memory, and LPS was administered directly after conditioning and therefore one day before expression testing; LPS decreases freezing expression, indicating impairment of consolidation of contextual learning into memory (Dantzer et al., 2008; Pugh et al., 1998; Thomson and Sutherland, 2005). Therefore, CD40AB and LPS induce behavioral changes consistent with impaired learning about or memory of emotional stimuli, effects that could have translational relevance to the cognitive impairments that are common symptoms in SBS and MDD (DSM-5, 2013; ICD-10, 1994). However, it is important to note that CS-US fear learning is primarily used as a test of emotional sensitivity to aversive stimuli. Indeed, CS-US learning is increased in MDD (Nissen et al., 2010), as it is in mice exposed to chronic psychosocial stress (Azzinnari et al., 2014; Fuertig et al., in prep.; Yu et al., 2011). That CD40AB does not impact on aversive sensitivity *per se* was demonstrated by the negative finding in the aversive-odor test. The synaptic plasticity, for example in the form of long-term potentiation, that is proposed to underlie CS-US and context-US learning-memory is dependent on optimal functioning of glutamate signaling (Johansen et al., 2011; Maren et al., 2013). Glutamate signaling could be impacted by CD40AB and LPS, including via activation of the KYN pathway, in particular increased brain levels of KYNA (a glutamate receptor antagonist) or QUIN (a NMDA glutamate receptor agonist) (Felger and Miller, 2012; Haroon et al., 2012). This hypothesis was not investigated in the present study and will be the focus of a future study.

There was no effect of CD40AB on running in the electrified treadmill test at post-injection day 5, suggesting that it did not impact on fatigue due to enforced physical activity. Chronic psychosocial stress, which activates inflammation, did induce a running deficit in this test (Azzinnari et al., 2014), and IL-1β administration induced a running deficit in a similar test (Carmichael et al., 2006). It is noteworthy that CD40AB did not induce increased IL-1β (see below),

and perhaps this is necessary for inflammation to lead to fatigue-like behavior. Fatigue, including physical fatigue, is a core symptom of SBS and MDD (Dantzer et al., 2014; Demyttenaere et al., 2005) and is particularly prominent in MDD comorbid with autoimmune disorders (Nikolaus et al., 2013).

4.2. The effects of CD40AB on pro-inflammatory cytokines and of TNF on sickness and saccharin drinking

The time courses of CD40AB effects on blood and brain levels of several pro-inflammatory cytokines and of tryptophan and its catabolites were established in the present study, to allow comparison with the time course of sickness and MDD-relevant behaviors. There is substantial evidence that SBS can be triggered by peripheral cytokine increases that stimulate synthesis and release of cytokines in the brain via neural and humoral pathways (for review: Dantzer, 2001). Based on studies using i.p. or intracerebroventricular administration of LPS or specific cytokines, central increases in IL-1β and TNF have been identified as the major instigators of sickness (Dantzer, 2001). These effects are proposed to be mediated in part by their deregulation of the expression levels of specific gene sets e.g. TNF effects on expression levels of clock genes (Cavadini et al., 2007). In the present study, at days 2-3 post-CD40AB, blood levels of TNF (see also Gast et al., 2013) and IFN-γ were increased markedly, whilst those of IL-1β and IL-6 were unchanged. At day 3, brain (hippocampal) levels of TNF (see also Gast et al., 2013), IL-1β and IL-6 were unaffected by CD40AB whilst there was a small increase in IFN-γ. Therefore, the present study provides evidence that the various behavioral states induced by CD40AB did not co-occur with altered brain protein levels of TNF, IL-1β or IL-6, and the IFN-γ increase was small.

Induction of SBS by CD40AB is dependent on increased peripheral TNF, as has been demonstrated using TNF receptor-1 knockout mice or etanercept, e.g. etanercept prevents

CD40AB induction of decreased activity and increased slow-wave sleep (Gast et al., 2013; Taraborrelli et al., 2011). Here we used etanercept to determine whether the observed reduction in saccharin drinking induced by CD40AB is also TNF dependent and, if yes, to establish the concomitant changes in cytokines and TRP catabolites. In CD40AB mice, etanercept completely blocked both sickness weight-loss and the decreased saccharin preference and consumption that otherwise occurred during and beyond sickness. In blood, etanercept led to marked attenuation of the IFN-γ increase. This was likely due to the increase in blood TNF - itself unaffected by etanercept - being without biological activity given that it was bound by etanercept. In hippocampus, etanercept actually increased TNF in CD40AB mice, and was without effect on IFN-γ, IL-6 and IL-1β. Therefore, this experiment provided further evidence that increased bio-active, peripheral TNF is essential for CD40AB induction of sickness, and that elevated central levels of the pro-inflammatory cytokines TNF, IFN-γ, IL-6 and IL-1β do not underlie this. It also demonstrated that the acute increase in plasma TNF is necessary to mediate the CD40AB-induced reduction in saccharin drinking.

4.3. The effects of CD40AB-TNF on kynurenines and their relevance to sickness and saccharin drinking

Regarding the finding that CD40AB transiently increased blood TNF and IFN-γ, using bacterial inoculation (Bacillus Calmette-Guérin) it has been demonstrated that increases in blood levels of these two cytokines are essential for inflammation to increase levels of the enzyme IDO (O'Connor et al., 2009a). In a non-inflammatory state, IDO is expressed at a low level by peripheral and brain immune cells, and its contribution to tryptophan metabolism is rather low. Following inflammation, peripheral and central IDO expression increase markedly (Dantzer et al., 2008). Although IDO was not measured in the present study, indirect evidence for activation by CD40AB

was provided by the increase in levels of kynurenines, and direct evidence for activation was provided by pharmacological IDO inhibition preventing this increase. In blood, CD40AB induced either no change or a moderate decrease in TRP, whilst KYN was increased at days 2-8, 3-HK at day 2, QUIN at days 2-7, KYNA was unchanged, and 5-HT was decreased at days 2-8. These effects are consistent with CD40AB triggering IDO activation, a consequent increase in KYN, and KYN catabolism by KYN-monooxygenase and kynureninase to QUIN (Felger and Miller, 2012; Mellor and Munn, 2004; Schwarcz et al., 2012). Etanercept blocked each of these CD40AB effects, demonstrating that TNF is an essential mediator of KYN pathway activation in the periphery by CD40AB. In brain (hippocampus, mPFC, dorsal raphe nucleus), following CD40AB there was an increase in TRP (hippocampus only), KYN was increased at days 2-8, 3-HK was increased at days 2-6, and 5-HT was unchanged. It is estimated that 60% of brain KYN is derived from the blood (Fukui et al., 1991; Gal and Sherman, 1978), and the respective levels suggest that blood KYN contributed directly to brain KYN in the present study: thus the time course of increased brain KYN largely paralleled that in blood, and increases in brain pro-inflammatory cytokines were minor and presumably insufficient to support increased microglial IDO expression. The increase in 3-HK in brain was more prolonged than in blood suggesting it was mainly produced from KYN in the brain. Etanercept blocked or at least markedly attenuated each of these CD40AB effects on brain kynurenines, as well as the increase in QUIN that was quantifiable in striatum. In LPS-injected mice, at 1 day post-treatment, brain TRP was increased, KYN was increased in blood and brain and, in contrast to the present study, brain 5-HT was increased (O'Connor et al., 2009b).

Therefore, there was a close temporal relationship between the durations of the CD40AB stimulatory effect on brain KYN levels and its inhibitory effect on saccharin drinking, i.e. 7-8 days, and both of these effects were blocked by etanercept. Based on these findings we investigated for a causal relationship between KYN pathway stimulation and decreased saccharin drinking by

repeated administration of an IDO inhibitor. The IDO inhibitor was efficacious in blocking CD40AB activation of the KYN pathway, in both blood and brain. However, the subsequent absence of elevated kynurenines was without effect on behavior, both in terms of sickness and saccharin drinking. These findings provide substantive evidence that CD40-TNF activation of the kynurenine pathway is not necessary for induction of either SBS or decreased saccharin drinking. It is important to compare these negative findings with those obtained with LPS. Both peripheral and ICV LPS-injected mice exhibited decreased sucrose preference in the two-bottle test and decreased activity in anti-depressant screening tests, namely the forced swim test (FST) and tail suspension test (TST) (Lawson et al., 2013; O'Connor et al., 2009b). Chronic pre-treatment with the IDO inhibitor 1-methyl tryptophan (1-MT) decreased peripheral-LPS-induced increase in the KYN/TRP ratio in blood and brain (O'Connor et al., 2009b). 1-MT was without effect on sickness induced by either peripheral or ICV LPS (Lawson et al., 2013; O'Connor et al., 2009b; Salazar et al., 2012), but nonetheless attenuated/blocked LPS effects in the sucrose preference test (Lawson et al., 2013; Salazar et al., 2012) and blocked LPS effects in the FST and TST (Lawson et al., 2013; O'Connor et al., 2009b). Therefore, neither the current IDO inhibitor administered after CD40AB nor 1-MT administered prior to and following LPS prevents SBS. With respect to behavioral end points, IDOInh was ineffective against CD40AB-induced decreased saccharin drinking, whereas 1-MT was effective against LPS-induced decreased sucrose preference and decreased activity in FST and TST.

4.4. Conclusions and outlook

CD40L:CD40 binding leading to immune-activation is a major aetio-pathological factor in infection and autoimmune disorders. The present study provides evidence that prolonged activation of the IDO-KYN pathway in periphery and brain is one component of the immune-inflammation complex

induced by CD40 signaling, and that this effect is triggered by an acute increase in blood TNF; the latter co-occurs with increased blood IFN-γ (present study) and increased blood M-CSF and IL-18 (Müller et al., in press). This study also identifies relatively long-lasting behavioral effects of CD40 activation in terms of decreased saccharin drinking and impaired Pavlovian fear learning. Despite the similar temporal extents of the KYN pathway activation and these behavioral deficits, combining IDO inhibition with CD40 activation was ineffective in blocking either the classical sickness measure of weight loss or the decrease in saccharin drinking. There is evidence that activation of the KYN pathway occurs in MDD (Kim et al., 2012). It has also been observed in mice exposed to chronic psychosocial stressors (Agudelo et al., 2014; Fuertig et al., in prep.; Laugeray et al., 2010; Miura et al., 2011), manipulations that lead to decreased gustatory reward sensitivity but, in contrast to CD40AB, increased aversion sensitivity including higher levels of fear conditioning (Azzinnari et al., 2014; Fuertig et al., in prep.; Tye et al., 2013; Yu et al., 2011). Given that CD40AB and psychosocial stress exert different constellations of behavioral effects, it is important to consider whether CD40AB effects constitute an "extended-SBS" rather than MDD-relevant psychopathology. It might indeed be parsimonious to interpret decreased saccharin drinking/motivation and impaired fear conditioning as an extended SBS. This is an important issue, particularly given that extended-SBS and MDD-relevant psychopathologies could have common or different aetio-pathophysiologies. In this respect, studies of the effects of IDO inhibition in rodent psychosocial stress models will be important, and it is noteworthy that the IDO inhibitor used in the present experiment reversed the increase in conditioned fear expression induced in mice by chronic psychosocial stress (Fuertig et al., in prep.). Furthermore, recently, colony-stimulating factor-1 receptor (CSF1R) was identified as a mediator of CD40AB induction of SBS via a pathway involving polarization of inflammatory monocytes in liver (Müller et al., in press); depletion of these hepatic macrophages by selective blocking of CSF1R leads to a shift in inflammatory

monocytes towards a resolution-like phenotype with concomitant expression of IL-10 that prevents SBS (Müller et al., in press). A future aim will be to assess the involvement of this pathway in the causation of what might be most parsimoniously regarded as "extended-SBS" behaviors, such as the constellation of decreased reward drinking and impaired fear learning, induced by CD40 immune-activation. Finally, the present study measured only a small number of the factors that could potentially mediate the observed acute and extended SBS effects of CD40AB. For example, the cytokines TNF, IL-1, IL-6 and IFN-γ were measured, but other cytokines including IL-10, IL-12, IL-17 and IL-18 have been reported to be increased within 48 h after CD40 activation (e.g. Müller et al., in press; for review: Chatzigeorgiou et al., 2009) and active in the brain. Tissue macrophages, inflammatory monocytes, dendritic cells and B-cells are among the cell types activated by CD40 signaling that could stimulate immune-inflammation contributing to the extended SBS. For T-cells, there is also a complex inter-relationship with the IDO-KYN pathway: for example, IDO-induced TRP depletion suppresses CD8+ T-cells (e.g. Uyttenhove et al., 2003). KYN is an endogenous ligand of the aryl hydrocarbon receptor (AHR) that is expressed in T-cells, and KYN-AHR binding leads to increased regulatory T-cell activity thereby reducing the immune response (for review: Julliard et al., 2014). Accordingly, suppression of the IDO-KYN pathway would be expected to have protective but also destabilizing effects on T-cell immunomodulation, and the potential contribution of the latter to the lack of effect of IDOInh on the extended SBS will also require further investigation.

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Figure captions

Figure 1. Effects of CD40AB in the two-bottle saccharin versus water test conducted in the home cage or IntelliCage. In both experiments mice were given one 0.5% saccharin bottle and one water bottle on days -5 to 0 to determine baseline saccharin consumption and to prevent conditioned taste aversion of saccharin to CD40AB-induced sickness. Bottle positions were switched daily. Mice were injected with CD40AB (10 mg/kg) or IgG2a at 18:45 h on day 0. Values are mean +/- SD. *Home cage experiment* (A-C): The test was conducted daily at 08:00-16:00 h. (A) Absolute water consumption; on days -8 to -6 mice were given two bottles with water to determine baseline fluid consumption. (B) Absolute saccharin consumption. (C) Percent saccharin preference (saccharin solution consumed (g)/saccharin solution + water consumed (g)) x 100. *IntelliCage experiment* (D-F): The test was conducted continuously and the data shown are for the 12-h dark period (07:00-19:00 h) specifically. (D) Absolute number of visits at saccharin corner. (E) Absolute number of nosepokes at saccharin corner. (F) Absolute number of licks at saccharin bottle. In each measure, in two-way ANOVA there was a Treatment x Day interaction and asterisks indicate days on which there was a Treatment effect using Bonferroni *post hoc* test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2. Effects of CD40AB on locomotor activity, conditioning of a discrete tone or a context to electroshock, and reactivity to aversive odor. Values are mean +/- SD. Mice were injected with CD40AB (10 mg/kg) or IgG2a at 19:00 h on day 0. (A)-(B) *Locomotor activity and Habituation on day 4.* (A) Activity in neutral arena (a.u./min). (B) Percent time freezing in neutral arena. (C)-(D) *Tone CS conditioning*. (C) Percent time freezing during conditioning of a 20-sec tone CS to electroshock US on day 5. Per mouse mean freezing was calculated for pairs of consecutive CS-US trials. (D) Percent time freezing during test of expression of learned fear of the CS on day 6. Per mouse mean freezing was calculated for trios of consecutive CS trials. On each day, in two-way

ANOVA there was a Treatment x Trial interaction and asterisks indicate trials for which there was a Treatment effect using Bonferroni *post hoc* test: * $p < 0.05$, *** $p < 0.001$. (E)-(F) *Context conditioning*. (E) Percent time freezing during conditioning of context to electroshock US on day 4. Per mouse mean freezing was calculated for pairs of context-US inter-trial intervals (ITI). (F) Percent time freezing during test of expression of learned fear of the context on day 5. Per mouse mean freezing was calculated for trios of context trials. (G) *Unconditioned aversive odor.* Percent time freezing during exposure to trimethylamine. Per mouse mean freezing was calculated for trios of 60-sec trials.

Figure 3. Scatter plots and means for effects of CD40AB on plasma levels of TNF, tryptophan and metabolites across study days 2-12. Five CD40AB mice were measured per day; nine IgG2a mice were measured at days 2 (N=3), 4 (N=3), 8 (N=2) and 12 (N=1) and pooled. (A) Tumour necrosis factor. (B) Simplified tryptophan metabolic pathways: IDO, indoleamine-2,3-dioxygenase, KMO, kynurenine monoxygenase, KAT, kynurenine aminotransferase. (C) Tryptophan, (D) Kynurenine, (E) 3-OH-Kynurenine, (F) Quinolinic acid, (G) Kynurenic acid, (H) Serotonin. Asterisks and parentheses indicate days on which there was a Group effect of CD40AB versus IgG2a obtained using one-way ANOVA followed by Tukey's *post hoc* test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ versus IgG2a.

Figure 4. Scatter plots and means for effects of CD40AB on ventral hippocampus tissue levels of tryptophan and metabolites across study days 2-12. Mice were the same as those used to obtain the data in Fig. 5. 4-5 CD40AB mice were measured per day, and a total of 7-8 IgG2a mice were measured at various days post-treatment and pooled. (A) Tryptophan, (B) Kynurenine, (C) 3-OH-Kynurenine, (D) Serotonin. Asterisks and parentheses indicate days on which there was a

significant Group effect of CD40AB versus IgG2a obtained using one-way ANOVA followed by Tukey's *post hoc* test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ versus IgG2a.

Figure 5. Effects of co-administration of Etanercept with CD40AB on sickness and saccharin drinking behavior. Mice were injected with Saline-IgG2a, Etanercept-IgG2a, Saline-CD40AB or Etanercept-CD40AB at 19:00 h on day 0. Values are means. (A) Body weight, (B) Percent saccharin preference, (C) Absolute saccharin consumption, (D) Absolute water consumption. For each measure there was a two-way ANOVA Treatment x Day interaction, and letters indicate days on which there was a difference ($p<0.05$ or lower) between Saline-CD40AB mice and the group(s) indicated as obtained using Bonferroni test.

Figure 6. Effects of co-administration of Etanercept with CD40AB on tryptophan metabolites in dorsal striatum. Mice were sacrificed on day 3. Scatter plots and means for: (A) Tryptophan, (B) Kynurenine, (C) 3-OH-kynurenine, (D) Quinolinic acid. Asterisks and parentheses indicate cases where there was a Treatment effect obtained using one-way ANOVA followed by Tukey's *post hoc* test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Figure 7. Lack of effect of IDO inhibitor on CD40AB-induced changes in body weight, saccharin consumption and kynurenine plasma/ventral hippocampus levels. Mice were injected i.p. with CD40AB or IgG2a on day 0 (19:00 h) and from day 1 received twice per day (08:00 and 16:00 h) per os administration of IDOInh or VEH. (A) Body weight, (B) Saccharin consumption. Letters indicate days on which there was a difference ($p<0.05$ or lower) between CD40AB-VEH mice and/or CD40AB-IDOInh mice and the control group(s) indicated, obtained using Bonferroni test. Mice were sacrificed on day 5. (C) Plasma kynurenine, (D) ventral hippocampal kynurenine. Treatment

effect obtained using one-way ANOVA followed by Tukey’s *post hoc* test: * $p<0.05$, ** $p<0.01$, **** $p<0.0001$.

Figure 1

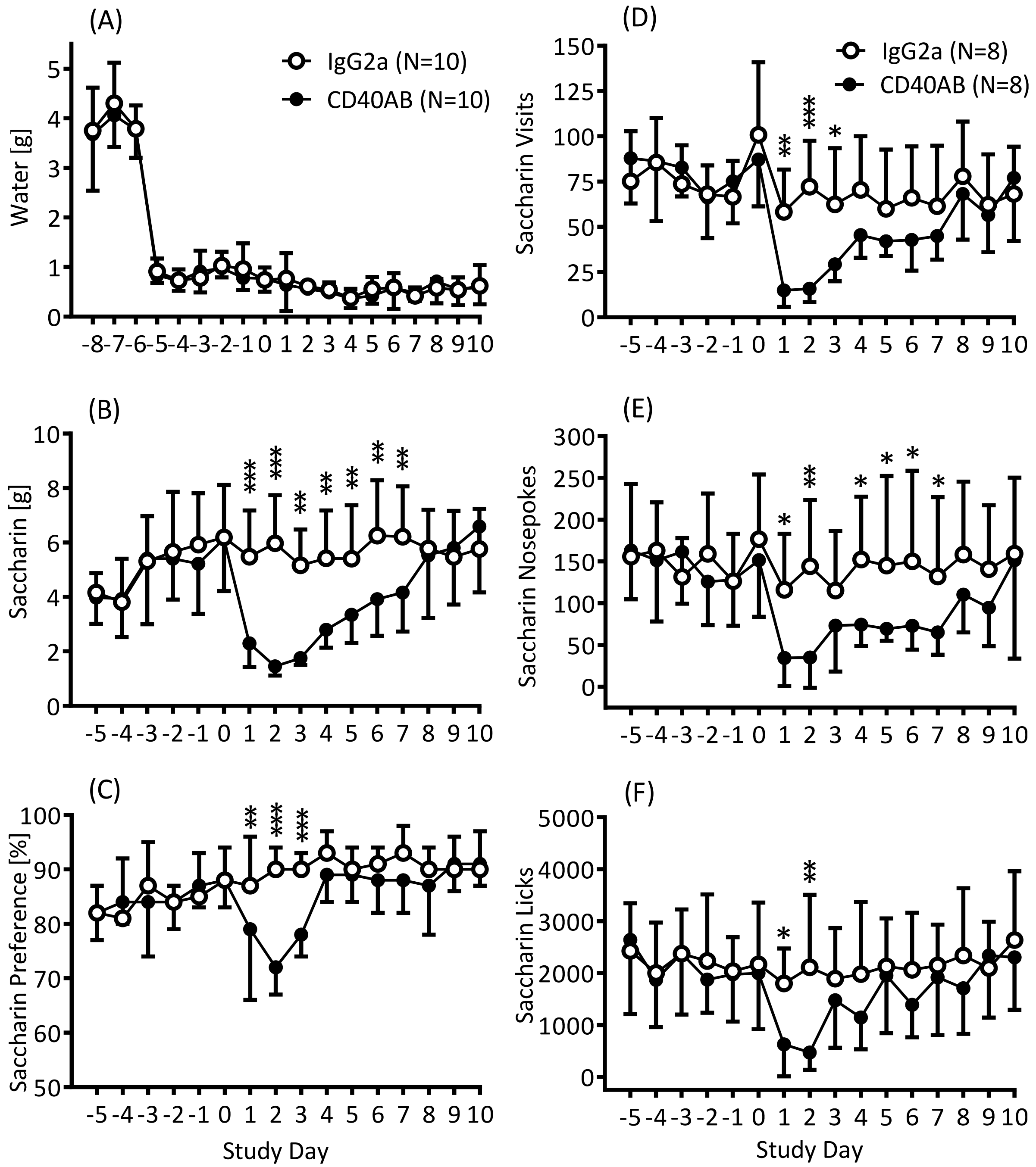
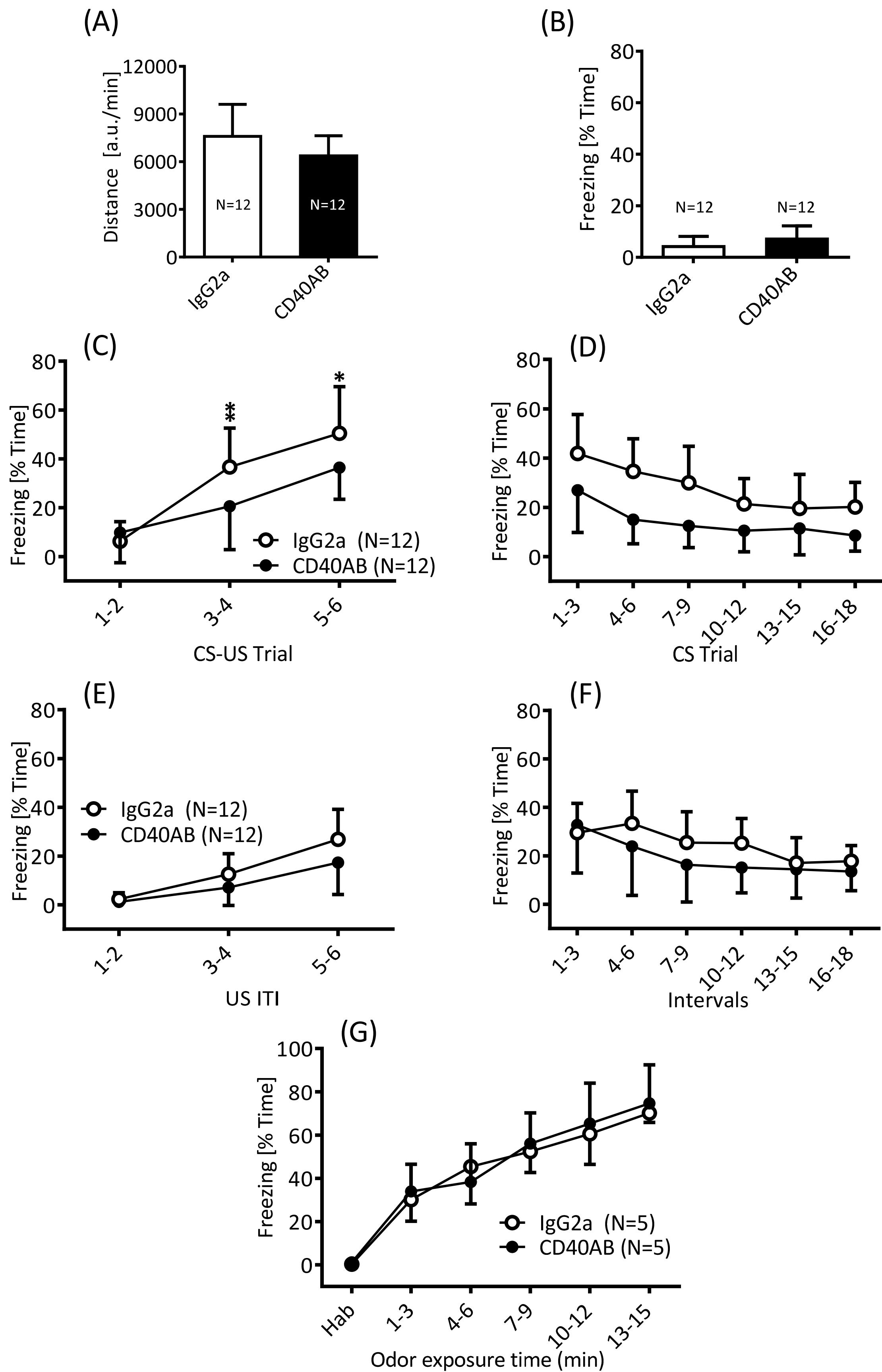


Figure 2



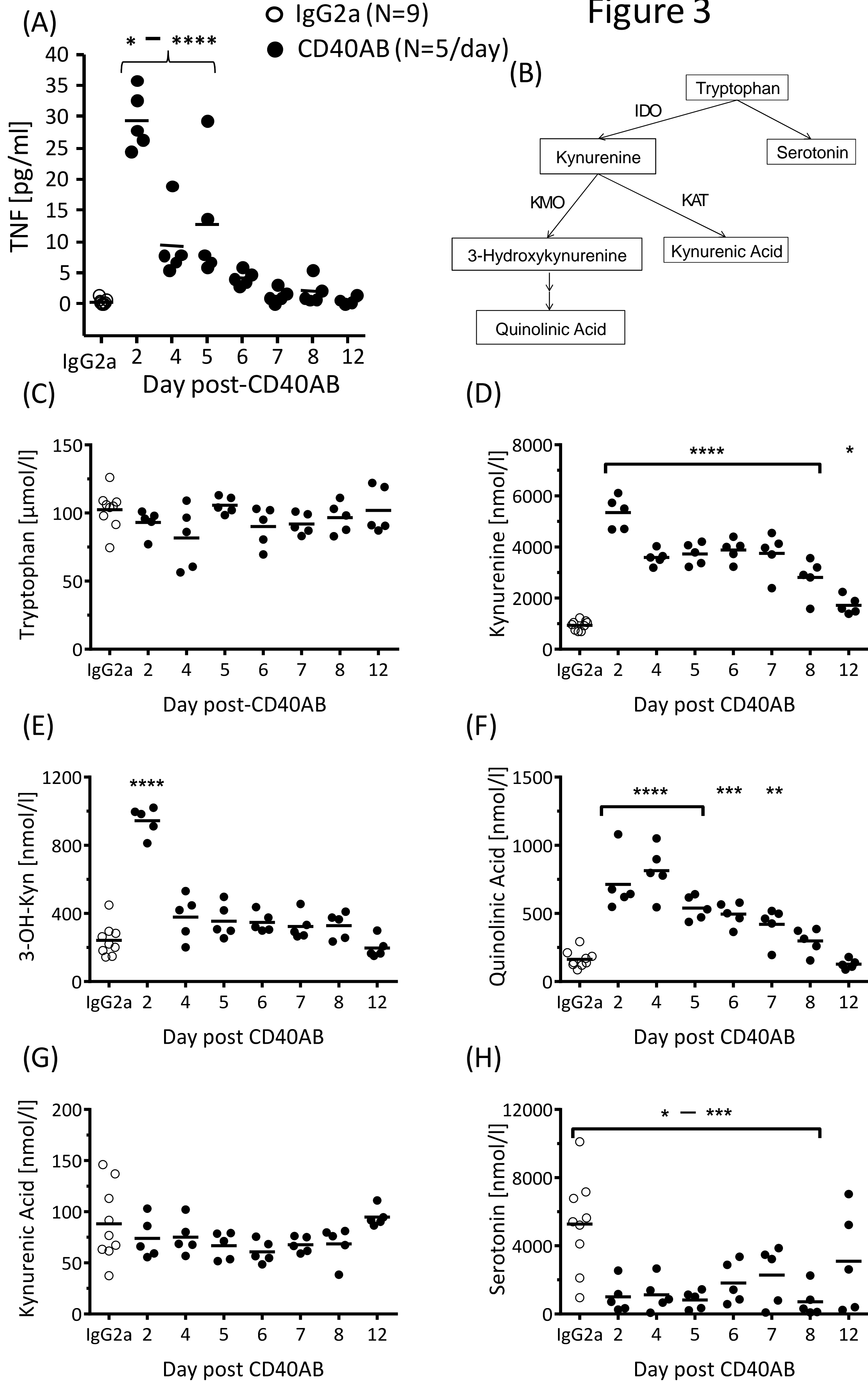


Figure 3
(A)
IgG2a (N=9)
CD40AB (N=5/day)
* – ****
TNF [pg/ml]
40
35
30
25
20
15
10
5
0
IgG2a 2 4 5 6 7 8 12
Day post-CD40AB
(B)
Tryptophan
IDO
Kynurenine
Serotonin
KMO
KAT
3-Hydroxykynurenine
Kynurenic Acid
Quinolinic Acid
(C)
Tryptophan [µmol/l]
150
100
50
0
IgG2a 2 4 5 6 7 8 12
Day post-CD40AB
(D)
Kynurenine [nmol/l]
8000
6000
4000
2000
0
****
*
IgG2a 2 4 5 6 7 8 12
Day post CD40AB
(E)
3-OH-Kyn [nmol/l]
1200
800
400
0
****
IgG2a 2 4 5 6 7 8 12
Day post CD40AB
(F)
Quinolinic Acid [nmol/l]
1500
1000
500
0
****
***
**
IgG2a 2 4 5 6 7 8 12
Day post CD40AB
(G)
Kynurenic Acid [nmol/l]
200
150
100
50
0
IgG2a 2 4 5 6 7 8 12
Day post CD40AB
(H)
Serotonin [nmol/l]
12000
8000
4000
0
* – ***
IgG2a 2 4 5 6 7 8 12
Day post CD40AB

Figure 4

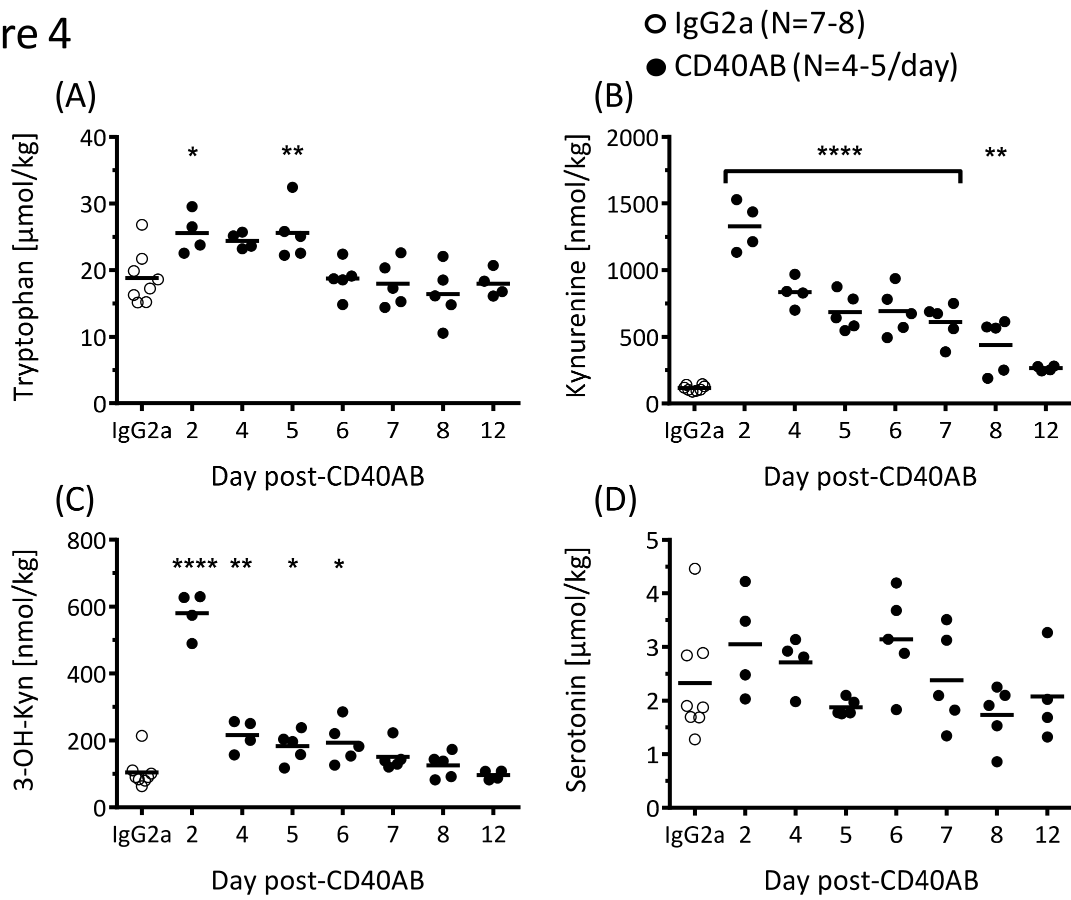


Figure 5

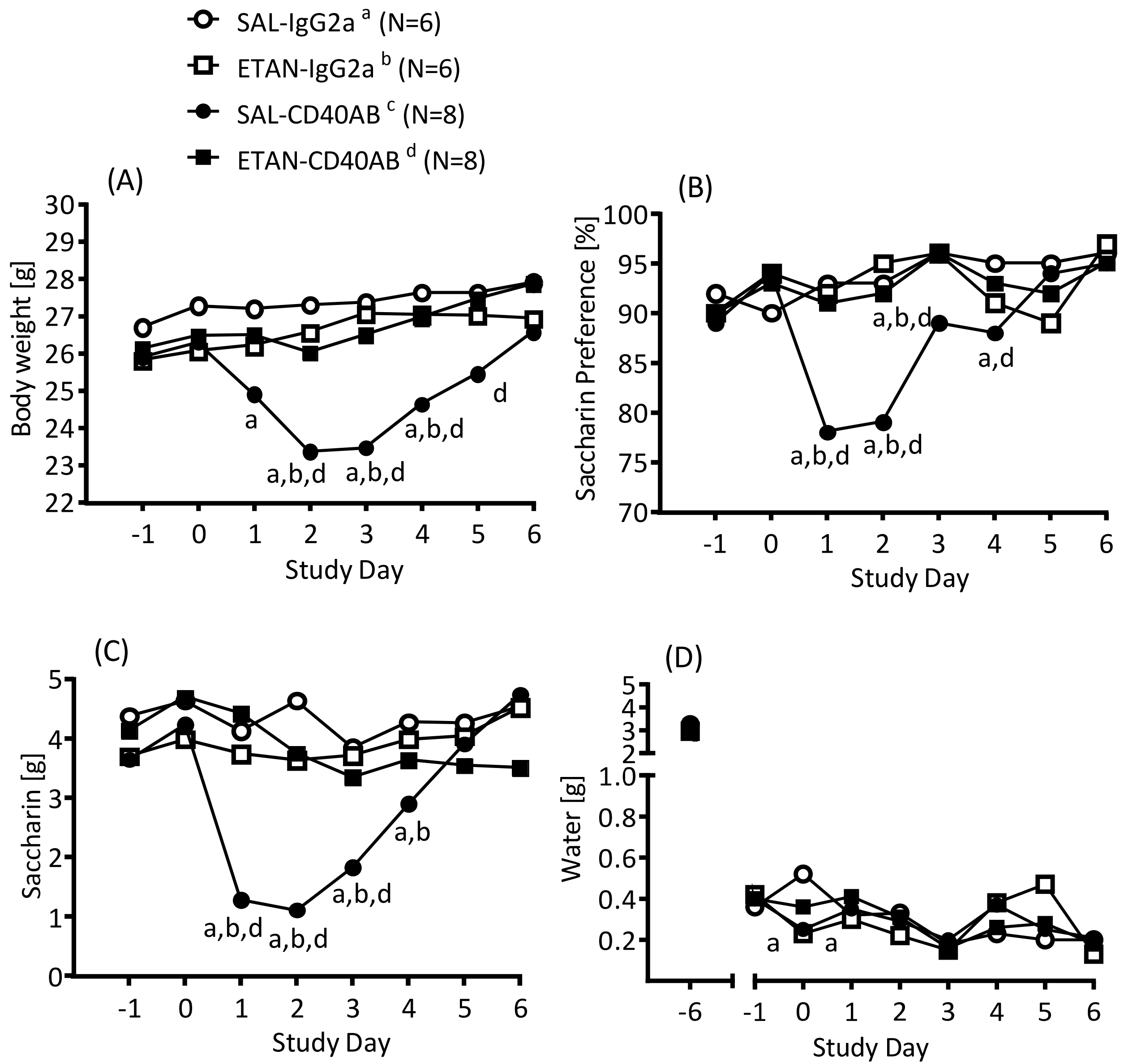


Figure 6

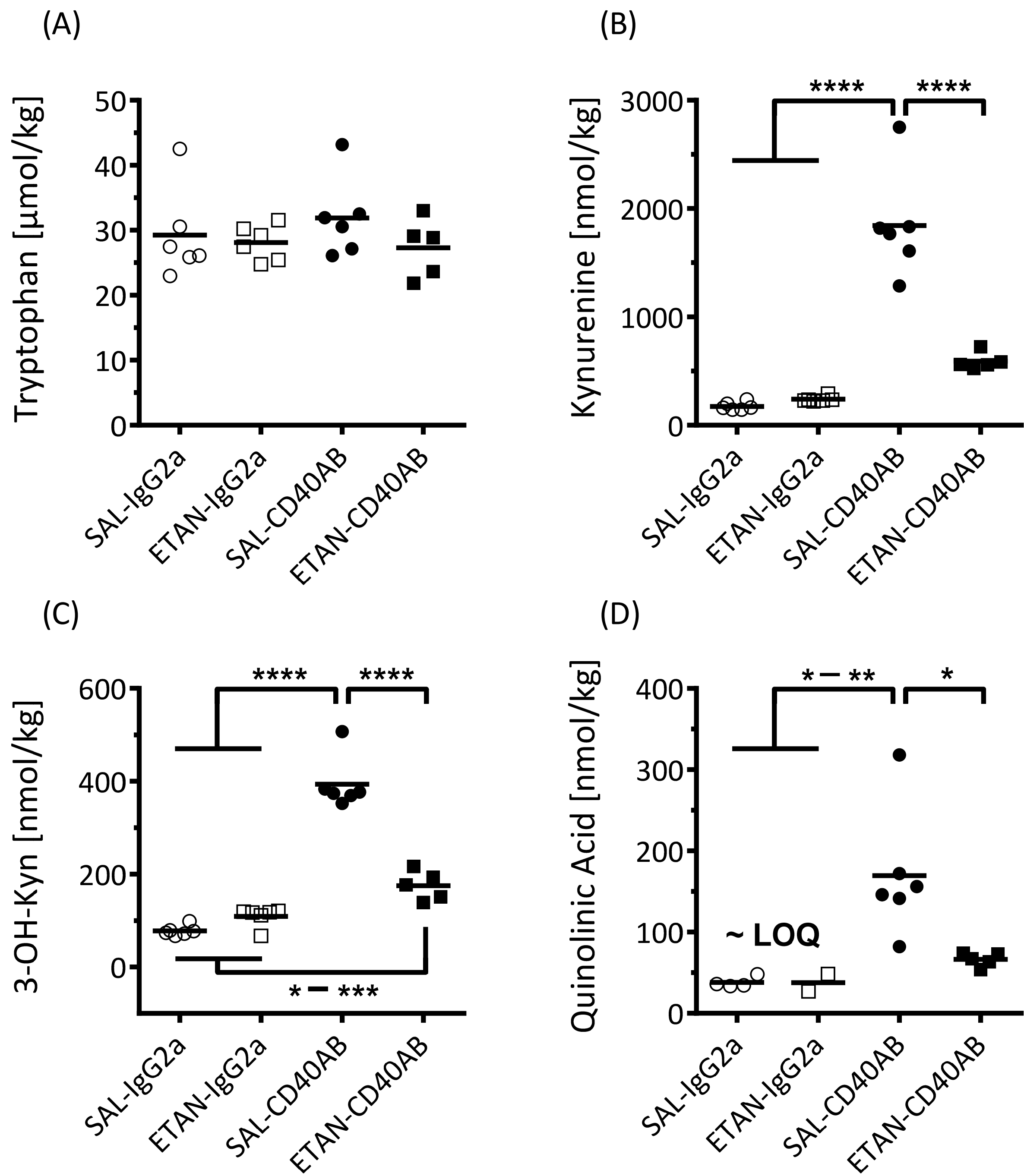
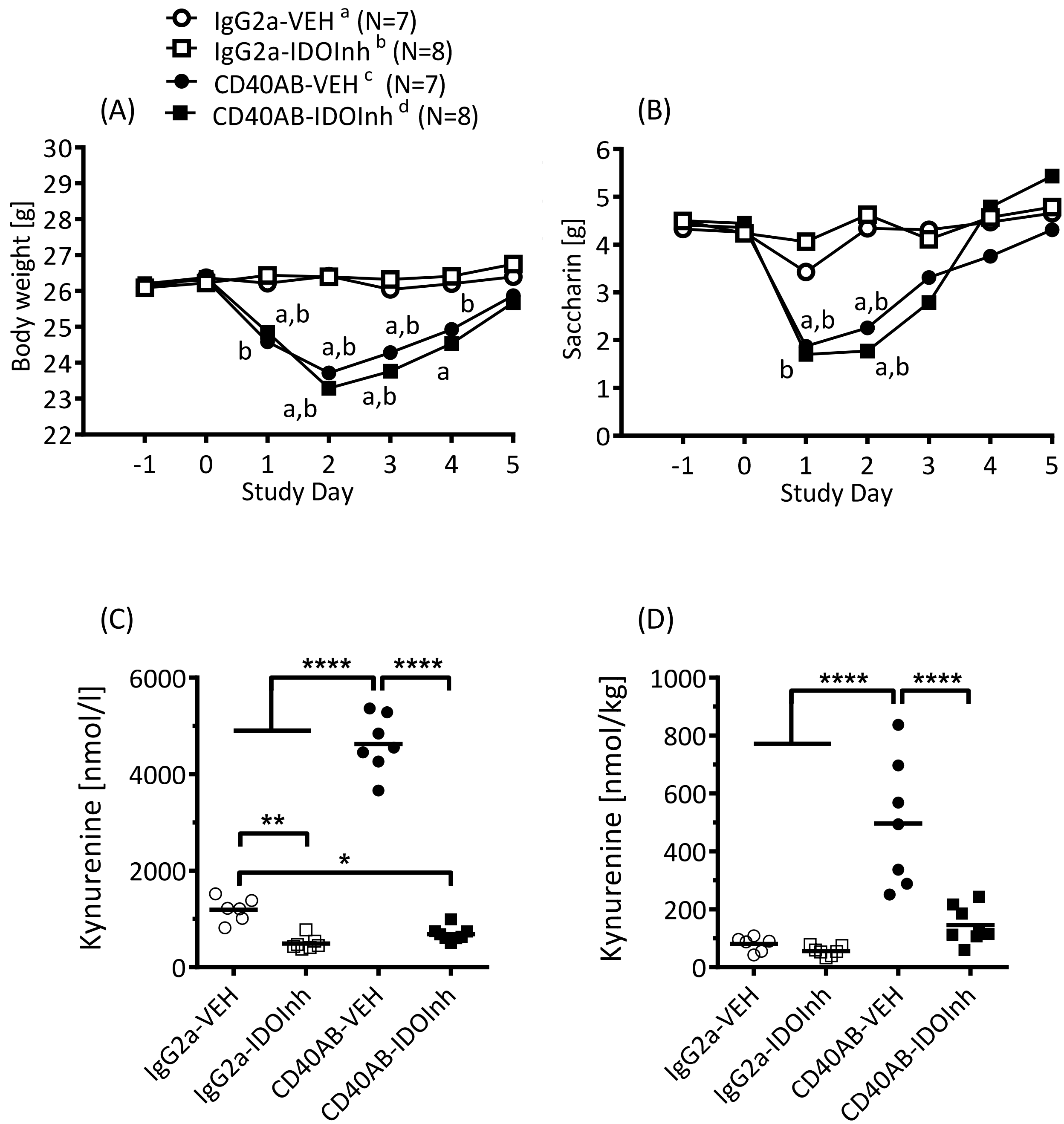


Figure 7



Supplementary Material

Figure captions

Figure S1. Scatter plots and means for effects of CD40AB on sickness measures across study days 2-12. Mice were injected with CD40AB (10 mg/kg) or IgG2a at 19:00 h on day 0. Per day, 5 CD40AB mice and 3 IgG2a mice were measured. For all end points there was no effect of day in IgG2a mice and data were pooled. (A) Body weight as percentage of pre-treatment baseline (day 0). (B) Food pellets consumed. (C) Percentage spleen weight relative to body weight. Absolute spleen weights (mg, mean±SD) were: IgG2a 67±6, CD40AB day 2 207±39, day 4 304±19, day 5 314±6, day 6 339±44, day 7 357±80, day 8 300±73, and day 12 128±28. Asterisks indicate days on which there was a significant Group effect of CD40AB versus IgG2a obtained using one-way ANOVA followed by Bonferroni *post hoc* test: * $p<0.05$, **** $p<0.0005$ versus IgG2a.

Figure S2. Saccharin preference effects of CD40AB in the IntelliCage operant choice test. The test was conducted continuously and the data shown are for the 12-h dark period 07:00-19:00 h specifically. (A) Percentage of visits at saccharin corner. (B) Percentage of nosepokes at saccharin corner. (C) Percentage of licks at saccharin bottle.

Figure S3. (A) Effects of co-administration of Etanercept with CD40AB on percentage spleen weight relative to body weight at day 6. (B) Effects of CD40AB ± repeated IDOInh or vehicle treatment on percentage spleen weight relative to body weight at day 5. **** $p < 0.0005$ for Treatment effect obtained using ANOVA and Bonferroni test.

Figure S4. Effects of co-administration of Etanercept with CD40AB on cytokine and tryptophan metabolites in plasma. Mice were sacrificed on day 3. Scatter plots and means for: (A) TNF, (B)

IFN-γ, (C) Tryptophan, (D) Kynurenine, (E) 3-OH-kynurenine, (F) Quinolinic acid. Asterisks and parentheses indicate cases where there was a Treatment effect obtained using one-way ANOVA followed by Tukey's *post hoc* test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Figure S5. Effects of co-administration of Etanercept with CD40AB on cytokine and tryptophan metabolites in hippocampus. Mice were sacrificed on day 3. Scatter plots and means for: (A) TNF, (B) IFN-γ, (C) Tryptophan. (D) Kynurenine, (E) 3-OH-kynurenine. Asterisks and parentheses indicate cases where there was a Treatment effect obtained using one-way ANOVA followed by Tukey's *post hoc* test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Figure S1

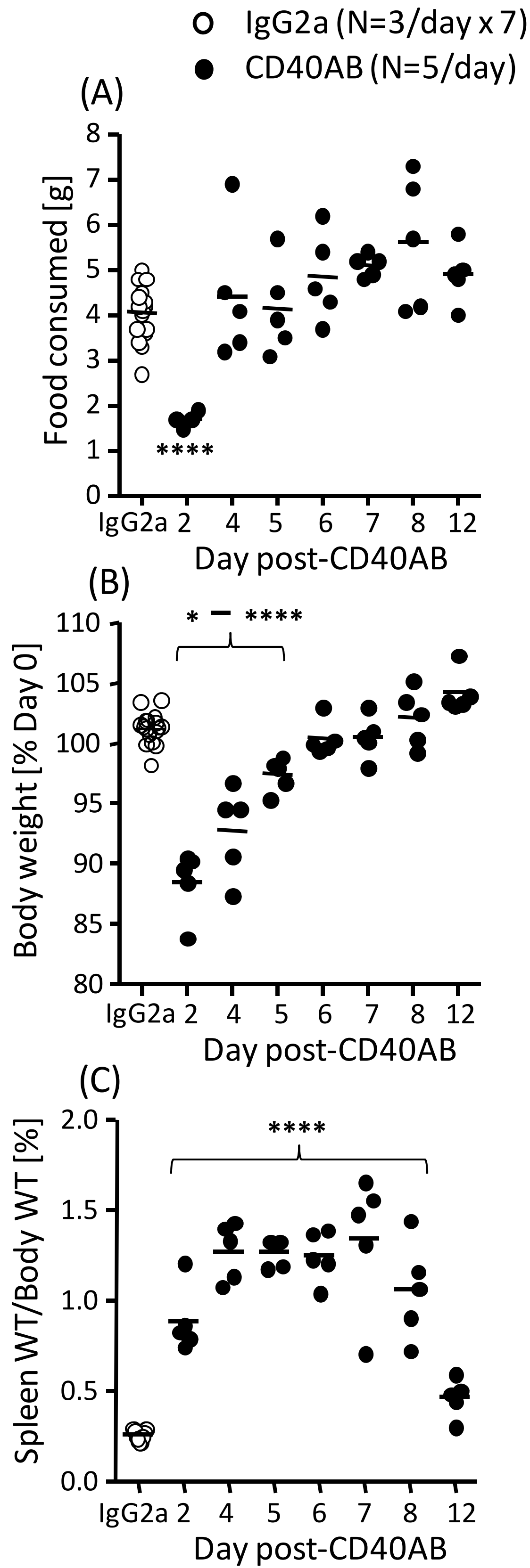


Figure S2

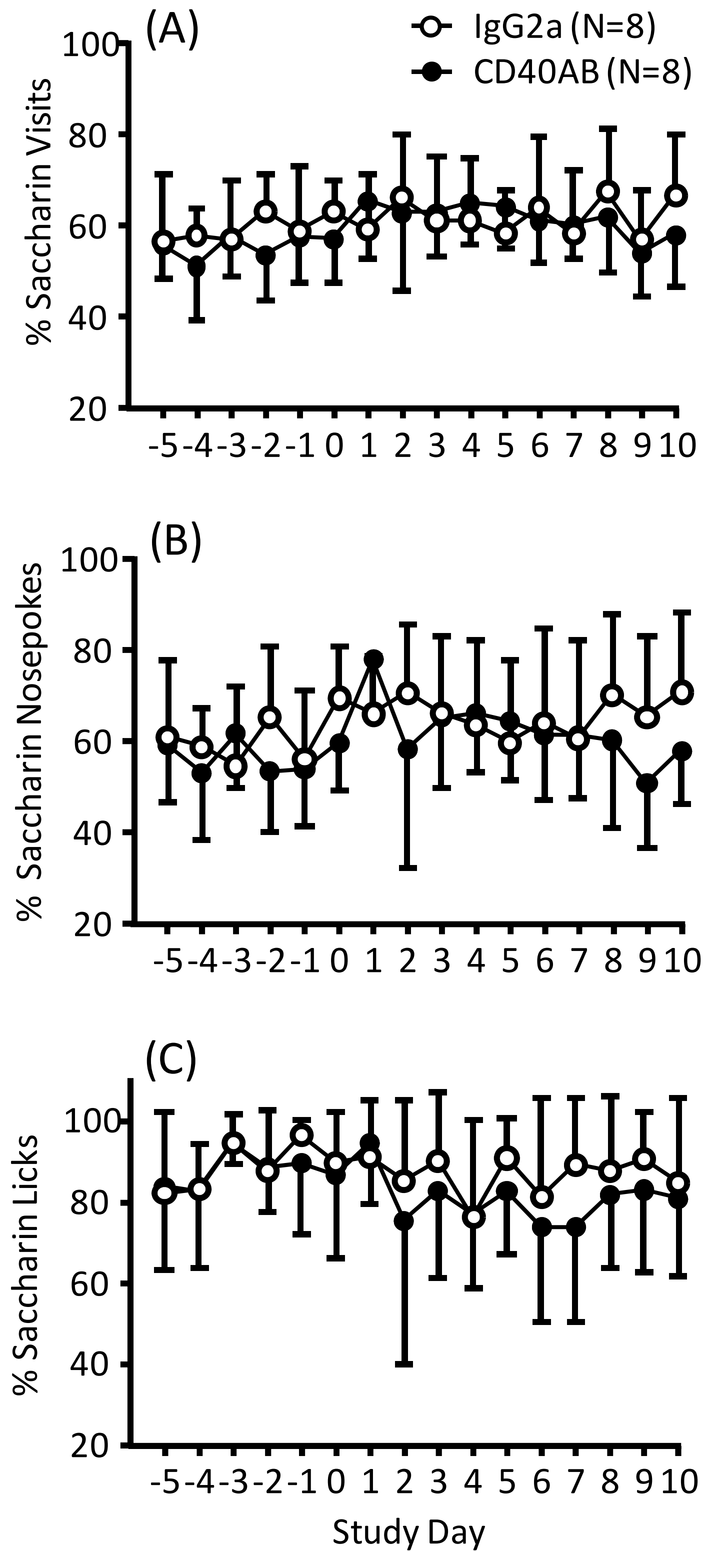
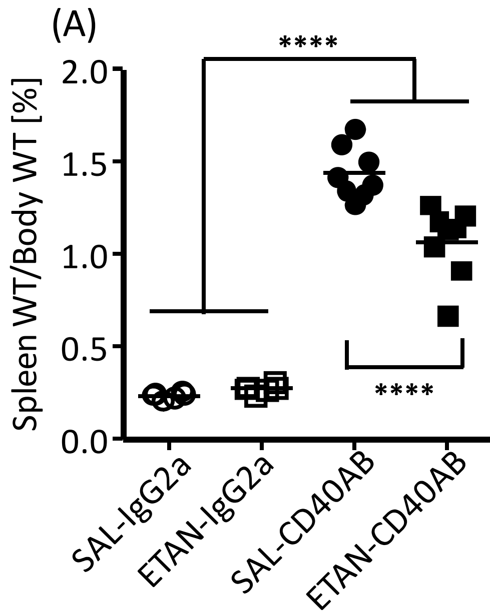


Figure S3



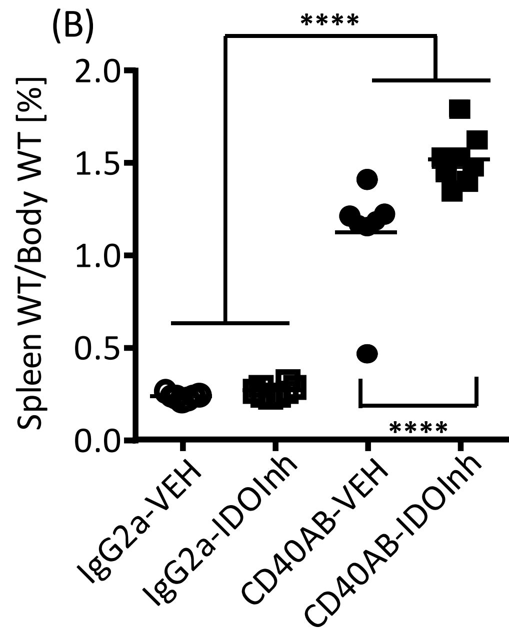


Figure S4

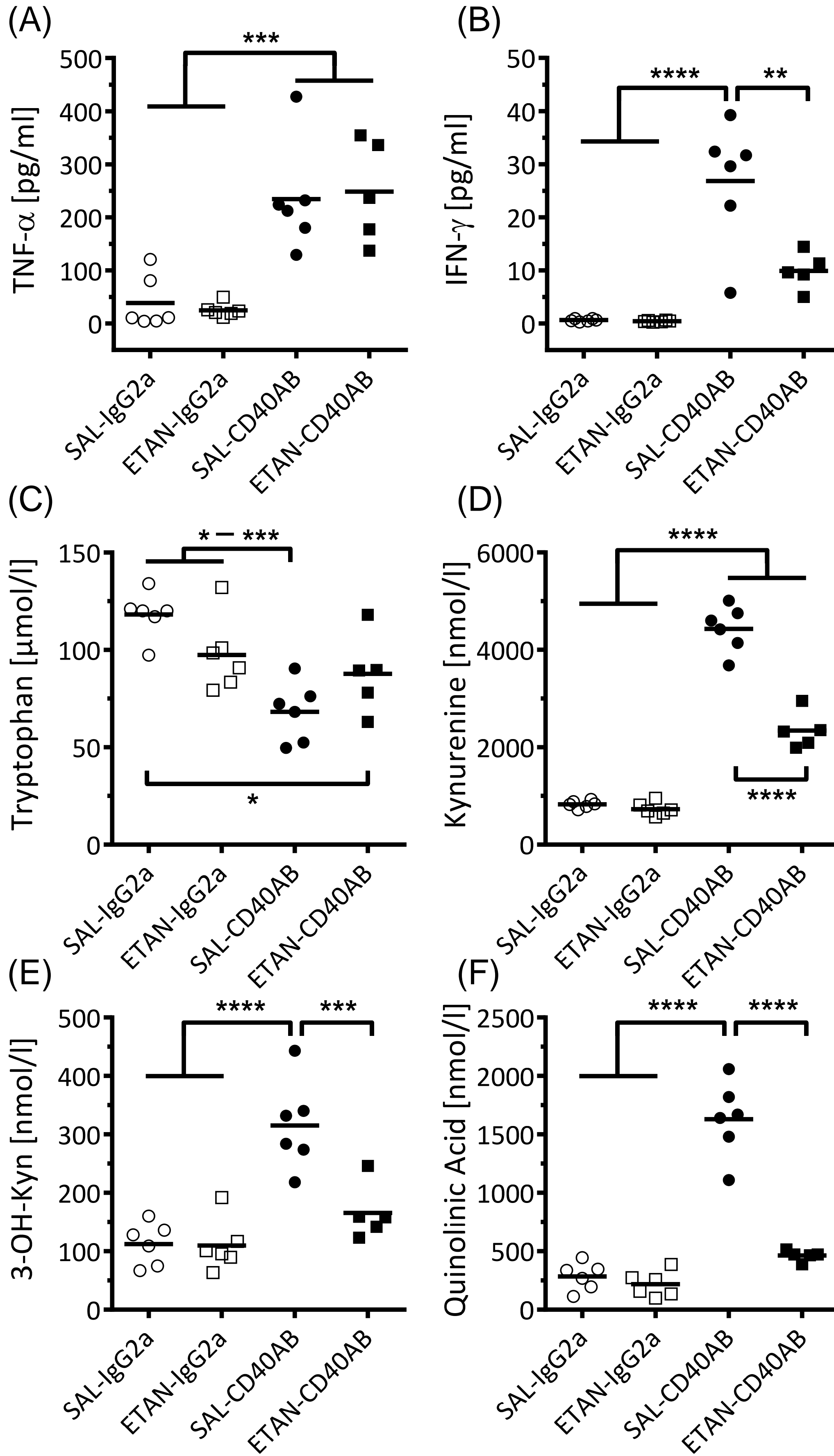


Figure S5

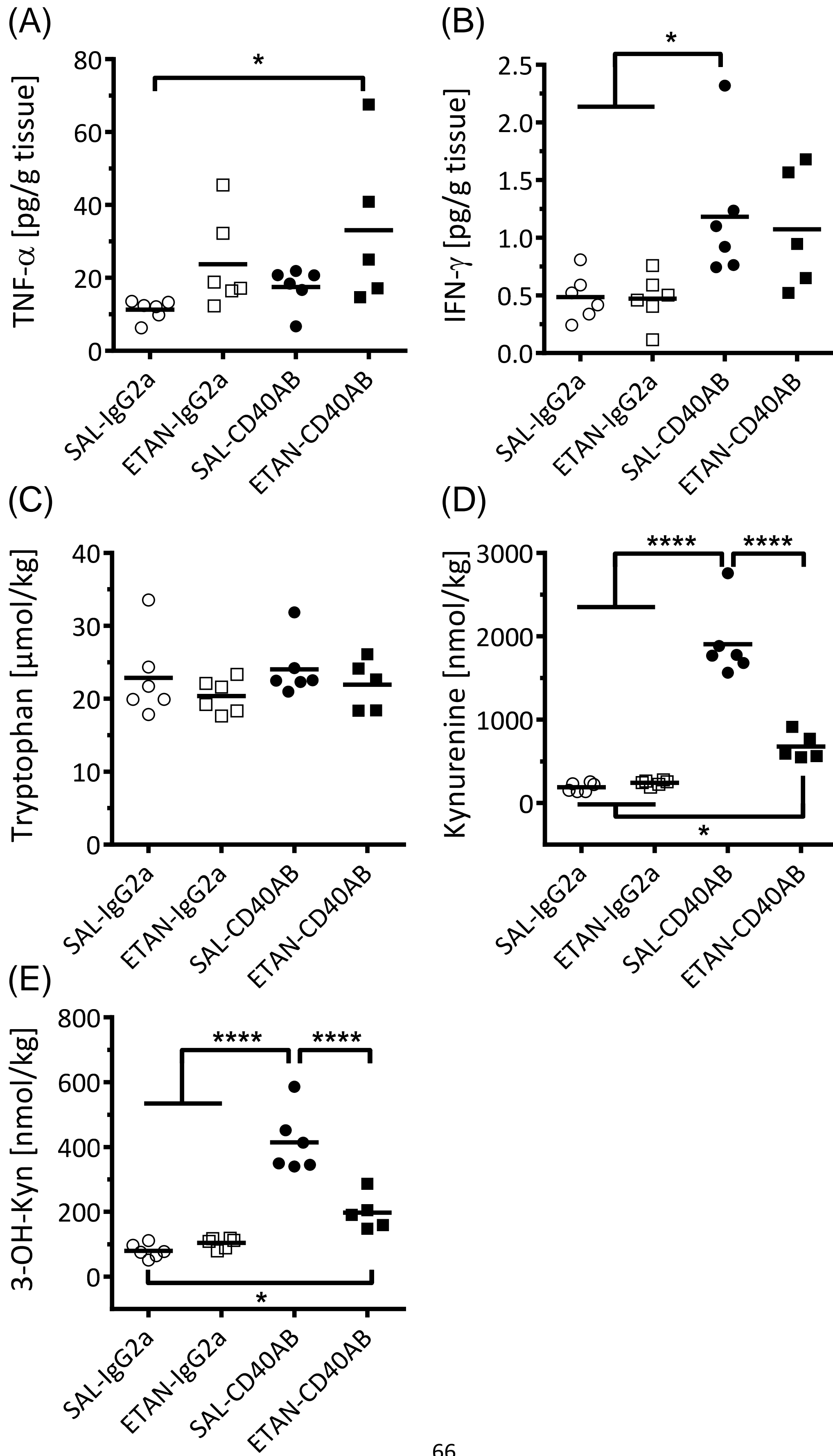


Table S1. Effects of CD40 agonist antibody on concentration (mean±SD) of tryptophan, kynurenines and serotonin in mouse brain regions

Region/ Metabolite	IgG2a	CD40 Day 2	CD40 Day 4	CD40 Day 5	CD40 Day 6	CD40 Day 7	CD40 Day 8	CD40 Day 12
ventral Hippocampus								
Tryptophan [μmol/kg]	18.9±4.0	25.6±3.1*	24.4±1.2	25.6±4.1**	18.7±2.7	18.0±3.5	16.4±4.3	18.0±2.0
Kynurenine [nmol/kg]	120±20	1330±180****	840±110****	690±140****	690±180****	610±140****	440±200**	260±20
3-OH-kynurenine [nmol/kg]	100±50	580±70****	220±50**	180±50*	190±60*	150±40	130±40	100±10
Serotonin [μmol/kg]	2.3±1.0	3.1±1.0	2.7±0.5	1.9±0.2	3.2±0.9	2.4±0.9	1.7±0.6	2.1±0.9
Medial prefrontal cortex								
Tryptophan [μmol/kg]	18.9±3.1	21.8±6.5	23.0±2.2	22.4±2.8	19.0±3.1	15.2±3.2	16.4±1.2	15.7±2.2
Kynurenine [nmol/kg]	130±40	1150±410****	840±150****	690±120****	640±160****	540±160***	390±180*	290±30
3-OH-kynurenine [nmol/kg]	80±20	360±100****	160±20**	110±20	140±40*	120±20	80±30	80±20
Serotonin [μmol/kg]	0.8±0.3	0.3±0.1	0.8±0.3	0.6±0.1	0.7±0.3	0.7±0.1	0.8±0.1	0.6±0.1
Dorsal raphe nucleus								
Tryptophan [μmol/kg]	11.9±3.9	14.3±3.0	19.8±5.5	19.9±8.7*	9.6±2.9	9.4±3.4	6.8±5.7	10.9±4.9
Kynurenine [nmol/kg]	100±30	920±70****	710±90****	630±140****	560±210****	440±110***	270±140	260±70
3-OH-kynurenine [nmol/kg]	70±20	270±40****	120±40	110±60	140±70*	90±20	90±20	70±10
Serotonin [μmol/kg]	2.5±1.6	4.4±2.2	2.5±1.8	3.4±1.5	4.5±1.5	3.5±2.1	3.1±2.1	3.5±2.1

IgG2a data were obtained with 7-8 mice with samples collected on various days, and CD40AB data were obtained with 4-5 mice per day post-treatment
* p<0.05, ** p <0.01, *** p<0.001, **** p<0.0001 for CD40AB mice at a specific day post-treatment versus IgG2a mice, based on ANOVA and Tukey's post hoc test.

Table S2. Effects of CD40 agonist antibody ± etanercept on concentration (mean±SD) of cytokines, kynurenic acid and serotonin in plasma on day 3

	IgG2a		CD40AB	
Analyte	Saline (N=6)	Etanercept (N=6)	Saline (N=6)	Etanercept (N=5)
IL-6 [pg/ml]	129.3±123.9	485.1±330.0*	163.0±90.8#	116.8±76.4#
IL-1β [pg/ml]	1.7±1.9	1.9±2.5	3.1±2.5	4.4±4.2
Kynurenic acid [nmol/l]	104.8±15.6	66.7±15.5**	63.7±7.6**	69.2±26.7*
Serotonin [µmol/l]	13.9±8.1	6.2±4.1	1.4±0.6**	5.0±2.9*

Two mice were excluded from the ETAN-CD40AB group as they exhibited weight-loss sickness indicative of ineffective etanercept administration

* p<0.05, ** p <0.01, versus SAL-IgG2a mice, # p<0.05 versus ETAN-IgG2a mice; based on ANOVA and Tukey's post hoc test

Table S3. Lack of effects of CD40 agonist antibody ± etanercept on concentration (mean±SD) of cytokines and serotonin in hippocampus and striatum on day 3

Region/	IgG2a		CD40AB	
Analyte	Saline (N=6)	Etanercept (N=6)	Saline (N=6)	Etanercept (N=5)
Hippocampus				
IL-6 [pg/g]	99±62	212±132	118±76	95±52
IL-1β [pg/g]	2.4±0.9	3.6±0.5	2.8±1.1	4.3±1.7
Serotonin [μmol/kg]	3.3±0.2	3.3±0.3	3.4±0.3	3.2±0.3
Striatum				
Serotonin [μmol/kg]	3.1±0.4	2.9±0.3	2.8±0.4	2.8±0.5

Two mice were excluded from the ETAN-CD40AB group as they exhibited weight-loss sickness indicative of ineffective etanercept administration

Table S4. Effects of CD40 agonistic antibody ± IDO inhibitor on concentration (mean±SD) of tryptophan, kynurenines and serotonin in plasma and hippocampus on day 5

Region/	IgG2a		CD40AB	
Analyte	Vehicle (N=6)	IDO inhibitor (N=6)	Vehicle (N=7)	IDO inhibitor (N=8)
Plasma				
Tryptophan [µmol/l]	137±35	94±29*	120±28	100±12
3-OH-Kynurenine [nmol/l]	144±55	55±34**	257±47$^{***/++++}$	33±14$^{***/\#\#\#\#}$
Quinolinic Acid [nmol/l]	248±95	134±66	675±242$^{****/++++}$	281±52$^{\#\#\#\#}$
Kynurenic Acid [nmol/l]	67±18	63±23	63±11	40±10$^{*/\#}$
Serotonin [µmol/l]	5.1±2.8	7.5±4.9	1.4±1.9^{+}	2.9±3.1
Hippocampus				
Tryptophan [µmol/kg]	11.4±1.6	11.7±2.7	15.0±4.2	21.1±9.3$^{*/+}$
3-OH-Kynurenine [nmol/kg]	75± 16 (3/6)[1]	46±10 (2/7)[1]	114±27 (6/7)[1]	49±26 (2/8)[1]
Serotonin [µmol/kg]	5.9±1.4	6.4±1.2	5.8±1.6	6.4±1.2

One IgG2a-VEH mouse and one IgG2a-IDOInh mouse were excluded from data analysis because of outlier values for plasma DA, QUIN and KYNA.

[1]Number of mouse samples with quantifiable 3-HK peaks

* p<0.05, ** p <0.01, *** p<0.001, **** p<0.0001 versus IgG2a-VEH mice, + p<0.05, ++++ p<0.0001 IgG2a-IDOInh mice; $^{\#}$ p<0.05, $^{\#\#\#\#}$ p<0.0001 versus CD40AB-VEH mice; based on ANOVA and Tukey's post hoc test