α-TLR2 antibody attenuates the Aβ-mediated inflammatory response in microglia through enhanced expression of SIGIRR

Derek A. Costello*, Dónal G. Carney & Marina A. Lynch

Department of Physiology & Institute of Neuroscience, Trinity College Dublin, Dublin 2, Ireland

Keywords: TLR2, SIGIRR, TIR8, Aβ, microglia, LTP, hippocampus, PPARγ, PI3 kinase, Akt

Acknowledgements: This work was funded by Science Foundation Ireland. DGC was the recipient of a Trinity College Postgraduate student award. The authors have no conflict of interest to declare.

*Corresponding author
Email : derek.costello@tcd.ie
Ph : +353 1 896 8476
Fax : +353 1 896 3545

ABSTRACT

The immunoregulatory function of Single-Ig-interleukin-1 related receptor (SIGIRR) is derived from its ability to constrain the inflammatory consequences of interleukin (IL)-1R and toll-like receptor (TLR)4 activation. This role extends to the brain, where SIGIRR deficiency increases the synaptic and cognitive dysfunction associated with IL-1R- and TLR4-mediated signalling. The current study set out to investigate the interaction between SIGIRR and TLR2 in brain tissue and the data demonstrate that the response to the TLR2 agonist, Pam_3CysSK_4 (Pam_3Cys_4), is enhanced in glial cells from $SIGIRR^{-/-}$ animals. Consistent with the view that β-amyloid peptide (Aβ) signals through activation of TLR2, the data also show that Aβ-induced changes are exaggerated in glia from $SIGIRR^{-/-}$ animals. We report that microglia, rather than astrocytes, are the primary glial cell expressing both TLR2 and SIGIRR. While Aβ increased TLR2 expression, it decreased SIGIRR expression in microglia. This was mimicked by direct activation of TLR2 with Pam_3Cys_4. We investigated the effect of an anti-TLR2 antibody (αTLR2) on the Aβ-induced inflammatory responses and demonstrate that it prevented the expression and release of the pro-inflammatory cytokines TNFα and IL-6 from microglia. In addition, application of αTLR2 alleviated the Aβ-mediated impairment in long-term potentiation (LTP) of hippocampal synaptic activity. The protective effects of αTLR2 were accompanied by an up-regulation in SIGIRR expression. We propose that a mechanism involving activation of PI3 kinase/Akt and the transcription factor peroxisome proliferator-activated receptor (PPAR)γ may facilitate this increase in SIGIRR. These findings highlight a novel role of SIGIRR as a negative regulator of TLR2-mediated inflammation in the brain.

INTRODUCTION

Toll-like receptors (TLRs) are a family of pathogen recognition receptors, ubiquitously expressed throughout the immune system. In recent years, an extensive body of evidence has

accumulated highlighting a role for TLRs in mediating innate immune responses in the brain and, potentially, their contribution to the pathogenesis of certain diseases (Carpentier et al., 2008; Hanke and Kielian, 2011; Lehnardt, 2010). The responses induced by TLR2 and TLR4 are the best characterized of the TLR family in the brain. Evidence of their cell-specific expression remains unclear to date (Laflamme and Rivest, 2001), and is likely to be confounded by differences in molecular signatures that have been identified in microglia from adults and neonates (Butovsky et al., 2014). However, expression has been reported on microglia and astrocytes *in vivo* and *in vitro* (Costello et al., 2011a; Hoffmann et al., 2007; Jack et al., 2005) and consistent with this, activation of TLR2 and TLR4 induces prominent inflammatory responses in the brain (Costello et al., 2011a; Hoffmann et al., 2007; Minogue et al., 2012; Nolan et al., 2003; Watson et al., 2010). Agonists like lipopolysaccharide (LPS), Pam_3Cys_4 and the endogenous ligand, high-mobility group box 1 (HMGB1), contribute to the detrimental consequences of neuroinflammation including impairment of hippocampal synaptic plasticity (Costello et al., 2011a; Costello et al., 2011b; Vereker et al., 2000a), epileptic activity (Auvin et al., 2010; Maroso et al., 2010) and cognitive dysfunction (Costello et al., 2011b; Mazarati et al., 2011).

β–amyloid peptide (Aβ), the constituent of neuritic plaques in Alzheimer's disease (AD), appears to be the primary mediator of the neuroinflammation that characterizes the disease (Akiyama et al., 2000; Lynch, 2014a; Wyss-Coray, 2006). Aβ induces glial activation (Liu et al., 2012; Lyons et al., 2012), which is associated with the synaptic and cognitive dysfunction typical of an inflammatory environment within the brain (Costello et al., 2005; Gallagher et al., 2013; Lyons et al., 2012). The evidence suggests a role for TLR2 and 4 in facilitating the inflammatory response to fibrillar Aβ in microglial cells (Jana et al., 2008; Reed-Geaghan et al., 2009) and Aβ-induced cognitive impairment (Vollmar et al., 2010). Consistent with this, when transgenic mice that overexpress amyloid precursor protein (APP)

were transplanted with microglia from TLR2-deficient mice, inflammation was reduced, as was Aβ-associated pathology (Liu et al., 2012). Expression of TLR2, and its co-receptor CD14, was enhanced in the brains of APP-overexpressing mice, and TLR2-positive cells were co-located with Aβ plaques in these animals and also postmortem tissue from AD patients (Letiembre et al., 2009).

In light of their innate immune function and ability to mediate rapid and profound responses, members of the TLR/interleukin-1 receptor family require tight regulation in order to maintain homeostasis. Single-Ig-interleukin-1 related receptor (SIGIRR/TIR8/IL-1R8) is a unique subtype of the family, as it does not possess either ligand-binding or signal transduction capacity (Thomassen et al., 1999). However, it exerts significant anti-inflammatory effects throughout the immune system (Garlanda et al., 2009; Riva et al., 2012), and SIGIRR deficiency enhances susceptibility to multiple autoimmune and inflammatory-associated conditions (Blok et al., 2014; Garlanda et al., 2007; Gulen et al., 2010; Lech et al., 2008; Veliz Rodriguez et al., 2012). SIGIRR negatively regulates IL-1R- and TLR4-mediated responses (Huang et al., 2006; Qin et al., 2005; Wald et al., 2003) and, in the brain of SIGIRR-deficient animals, the enhanced response to LPS was likely due to the increased expression of TLR4 (Costello et al., 2011b; Watson et al., 2010). Interestingly, hippocampal-dependent memory and long-term potentiation (LTP) were impaired in $SIGIRR^{-/-}$ mice, and the evidence suggested that this was mediated by an increase in TLR4-associated signalling and expression of HMGB1 (Costello et al., 2011b).

The interaction between SIGIRR and TLR2 remains relatively unexplored, and reports are somewhat contradictory. An investigation of monocytes from patients with sepsis indicated reduced TNFα production in response to the TLR2 agonist Pam_3Cys_4, which was associated with an increase in SIGIRR expression (Adib-Conquy et al., 2006), although SIGIRR did not regulate the TLR2-mediated inflammatory response in monocytes from patients following

cardiac arrest (Adib-Conquy et al., 2006). Monocytes from SIGIRR-deficient animals responded more profoundly to Pam_3Cys_4, whereas a similar interaction was not identified in renal tubular epithelial cells (Lech et al., 2007). More recently, the probiotic *L. jensenii* and Pam_3Cys_4 were reported to increase SIGIRR on porcine antigen presenting cells, in a TLR2-dependent manner (Villena et al., 2012). However the possibility that SIGIRR acts as a regulator of TLR2-mediated responses in the brain has not been assessed. Here, we set out to evaluate whether such regulation occurs and, specifically, to identify the role played by TLR2 and SIGIRR in the inflammatory changes induced by Aβ. Our findings demonstrate an inverse relationship between TLR2 and SIGIRR in microglia and indicate that Aβ negatively impacts on SIGIRR expression in a TLR2-dependent manner.

MATERIALS AND METHODS

Animals

In this study we used 1 day-old C57BL/6 mice (Harlan, UK) and $SIGIRR^{-/-}$ mice (a gift from Professor A. Mantovani, Istituto Clinico Humanitas, Istituto di Ricovero e Cura a Carattere Scientifico, Milan, Italy; (Garlanda et al., 2004)) to prepare glial cultures. Male C57BL/6 mice (5 month-old) and wildtype or TLR2-deficient mice (2-3 months-old; a kind gift from Professor P. Fallon, Trinity College Dublin) were used for electrophysiological experiments. All experiments were performed under license from the Health Products Regulatory Authority of Ireland, with local ethical approval (Department of Comparative Medicine, Trinity College Dublin) and in accordance with EU regulations. Animals were housed under controlled conditions (20-22°C, food and water *ad lib*) and maintained under veterinary supervision.

Preparation of primary mixed glia and enriched astrocyte and microglial cultures

Mixed glial cultures were prepared from 1 day-old C57BL/6 mice or SIGIRR$^{-/-}$ mice as previously described (Nolan et al., 2004). Briefly, brain tissue from 5-7 mice was dissected, roughly chopped and added to pre-warmed Dulbecco's modified Eagle's medium containing fetal bovine serum (FBS), penicillin and streptomycin (100U/ml) (cDMEM; Invitrogen, UK). Tissue was triturated, the suspension was filtered through a sterile mesh filter (40μm) and centrifuged (2000 rpm, 3 min, 20°C). The resulting pellet was resuspended in warmed cDMEM and cells seeded (1 x 10^5 cells/well) onto 6-well plates. Cultures were maintained for 12 days at 37°C in a 5% CO_2 humidified environment.

Enriched microglia and astrocytes were prepared from 1 day-old C57BL/6 mice as previously described (Costello and Lynch, 2013). Briefly, tissue was prepared as described above, with tissue from a minimum of 5 animals pooled to provide a homogeneous population of cells. Cells were seeded into 25cm^2 culture flasks and, after 24h, media was supplemented with granulocyte macrophage-colony stimulating factor (GM-CSF; 10ng/ml) and macrophage-colony stimulating factor (M-CSF; 10ng/ml; R&D Systems, UK), and cells were maintained in culture for a further 7-9 days. Non-adherent microglia were isolated by shaking (220 rpm, 2h, 37°C), tapping and centrifuging (2000 rpm, 3 min). The pellet was resuspended in cDMEM and the microglia were plated onto 24-well plates (0.6x10^5 cells/cm^2; 1x10^5/200μl) and maintained at 37°C in a 5% CO_2 humidified atmosphere for 2 days prior to treatment. Adherent astrocytes were incubated in trypsin-ethylenediaminetetraacetic acid (EDTA) (3ml/flask, 37ºC; Invitrogen, UK) for 3 min, and the digestion was inactivated by addition of cDMEM (6ml/flask). Cells were centrifuged (2000 rpm, 3 min) and pellets resuspended in cDMEM. Astrocytes were plated onto 6-well plates (0.3x10^5 cells/cm^2; 0.5x10^5/200μl) and incubation continued for 2 days (37°C in a 5% CO_2) prior to treatment.

Mixed glial cells from C57BL/6 and SIGIRR$^{-/-}$ mice were incubated for 24h in the presence or absence of Pam_3Cys_4 (100ng/ml; InvivoGen, UK) or a cocktail of $A\beta_{[1–40]}$ (4.5μM)

and $A\beta_{[1–42]}$ (5.5μM; Invitrogen, UK), and in some experiments enriched microglia and astroctytes were incubated with Aβ or Pam_3Cys_4 for 6h. To assess the role of TLR2 in mediating the effects of Aβ, microglia were incubated with an αTLR2 antibody (2.5μg/ml; Hycult, UK) in the presence or absence of Aβ, and the modulatory effects of the PI3 kinase inhibitor LY294002 (LY; 10μM; Calbiochem, US) or the PPARγ antagonist GW9662 (GW; 20μM; Sigma-Aldrich, UK) were examined. For all experiments involving αTLR2, mouse IgG1a (2.5μg/ml; Santa Cruz Biotechnology, US) was included as an isotype control. An additional set of experiments were carried out by pre-incubating microglia in the presence or absence of an αCD16/CD32 antibody (10μg/ml, 90min; BD Pharmingen, UK) prior to application of either αTLR2 or IgG1a. Supernatant samples were stored at -20°C for later analysis of TNFα and IL-6. Cells were prepared for Western immunoblot or quantitative PCR analysis, as described below. Treatments were carried out in duplicate or triplicate, and mean results from at least 3 independent experiments are presented.

Electrophysiological analysis

Hippocampal slices were obtained from male C57BL/6 mice (5 month-old) and wildtype and $TLR2^{-/-}$ mice (2-3 month-old) as previously described (Costello et al., 2011b). In brief, hippocampal slices (400μm) were prepared using a McIlwain tissue chopper, equilibrated at room temperature for ≥1h, and incubated for 1h with either αTLR2 (2.5μg/ml; 1.4ml) or IgG1a (2.5μg/ml; 1.4ml) in oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 1.25 KCl, 2 $CaCl_2$, 1.5 $MgCl_2$, 1.25 KH_2PO_4, 25 $NaHCO_3$, and 10 D-glucose. Each slice was transferred to a submerged recording chamber and continually perfused (2-3ml/min) with oxygenated aCSF, at room temperature (23-24°C).

Excitatory post-synaptic potentials (EPSPs) were evoked by stimulating the Schaffer collateral-commissural pathway at 0.033Hz (0.1ms duration) using a bipolar tungsten stimulating electrode (Advent Materials, UK). Extracellular EPSPs were recorded from the CA1 stratum radiatum using a monopolar recording electrode, pulled from borosilicate glass capillary tubes (Harvard Apparatus, US) and filled with aCSF. The stimulus intensity was adjusted to produce a response ~50% of maximal EPSP amplitude as determined from an input–output curve for each experiment. A stable baseline of at least 20-40 min was recorded prior to application of theta-burst stimulation (TBS), which consisted of 10 trains (4 pulses at 100Hz) repeated at 5Hz. Some experiments were carried out in the presence of $A\beta_{[1–40]}$ (200nM), applied 40 min prior to TBS, as previously described (Lyons et al., 2012). Evoked EPSPs were normalised to the slope recorded in the 5 min period prior to LTP induction, and LTP was measured as a mean value of the final 5 min of recording (55-60 min post-TBS). Data are presented as mean percentage EPSP slope ± SEM in 3-4 slices, and for clarity of illustration, error bars are included to correspond with every 2 min of recording. Sample EPSP traces represent an average of 4 consecutive EPSPs, taken immediately prior to TBS, and 60 min following LTP induction. All data were acquired using WinWCP v4.0.7 software (Dr J. Dempster, Strathclyde, UK). Hippocampal slices not used for recording were incubated in either αTLR2 or IgG1a (as above) and stored for later mRNA analysis.

Quantitative real-time PCR

Total RNA was extracted from cultured mixed glia, microglia, astrocytes and hippocampal tissue using a NucleoSpin® RNAII isolation kit (Macherey-Nagel Inc., Germany) and cDNA synthesis was performed on total RNA using a High Capacity cDNA RT kit (Applied Biosystems, Germany). Real-time PCR was performed as described previously (Lyons et al., 2007) using an ABI Prism 7300 instrument (Applied Biosystems, Germany). The following

primers were used TLR2: Mm00442346_m1, SIGIRR: Mm00491700_m1, TNFα: Mm00443258_m1 and IL-6: Mm00446190_m1. Gene expression was calculated relative to β-actin as the endogenous control to give a relative quantity (RQ) value (2^{-DDCt}, where CT is the threshold cycle).

Assessment of supernatant concentrations of TNFα and IL-6

Supernatant concentrations of TNFα and IL-6 from cultured cell supernatant were assessed by enzyme-linked immunosorbent assay (ELISA), according to the manufacturers guidelines (R&D Systems, UK). Briefly, 96-well plates (Nunc, Denmark) were coated with goat anti-mouse TNFα or rat anti-mouse IL-6 antibody (50µl/well) overnight at room temperature, and blocked with 1% BSA in PBS (150µl/well) for 1h at room temperature. Supernatant samples and recombinant mouse TNFα or IL-6 standard (TNFα: 0-2000pg/ml; IL-6: 0-1000pg/ml; 50µl/well; in duplicate) were incubated overnight at 4ºC. Plates were washed (0.05% Tween20/PBS) and incubated in biotinylated goat anti-mouse TNFα or IL-6 antibody for 2h at room temperature. Plates were washed and incubated in the presence of HRP conjugated streptavidin in (1% BSA in PBS; 50µl/well) for 20 min. Substrate solution (50µl/well; 1:1 mixture of H_2O_2 and tetramethylbenzidine; Sigma-Aldrich, UK) was applied and incubated at room temperature in the dark for up to 20 min, and the reaction was stopped using 1M H_2SO_4 (25µl/well). Absorbance was read at 450nm using a BioTek Synergy HT microplate reader and data is presented as pg/ml.

Western immunoblotting

Cultured microglial cells treated with αTLR2 antibody or IgG1a (2.5µg/ml; 0-6h) were stored at -20ºC in lysis buffer (40µl; composition in mM: Tris-HCl 10, NaCl 50, $Na_4P_2O_7.H_2O$ 10, NaF 50, 1% Igepal, phosphatase inhibitor cocktail I and II, protease inhibitor cocktail; Sigma-

Aldrich, UK) for later assessment of Akt phosphorylation. Protein concentrations were equalised following MicroBCA protein quantification assay (Thermo Scientific, US). For analysis, samples were added to 4x sodium dodecyl sulphate (SDS) sample buffer (composition: Tris-HCl 100mM, pH 6.8, 4% SDS, 2% bromophenol blue, 20% glycerol; Sigma, UK) and heated to 70°C for 5 min. Protein samples (5μg) were separated on 10% standard SDS gels. Proteins were transferred to nitrocellulose membrane (Advansta, US) and blocked in Tris-buffered-saline containing 0.05% Tween® 20 (TBS-T) and 5% non-fat dried milk/TBS-T for 1h at room temperature. Membranes were incubated overnight at 4ºC with anti-pAkt (Ser^{473}) or anti-Akt (1:1000; Cell Signaling, US) antibodies in 2% non-fat dried milk/TBS-T, washed and incubated with a secondary anti-rabbit (1:5000; Jackson Immunoresearch, US) antibody in 2% non-fat dried milk/TBS-T for 1-2h. Immunoreactive bands were detected using WesternBright ECL chemiluminescent substrate (Advansta, US). Blots were stripped and re-probed using anti-β-actin (1:10000; Sigma, UK) and a peroxidase-conjugated secondary anti-mouse antibody (1:5000; Jackson Immunoresearch, US), both in 2% non-fat dried milk/TBS-T. Images were captured using the Fujifilm LAS-4000 imager. To quantify expression of the proteins, densitometric analysis was carried out using ImageJ (http://rsb.info.nih.gov/). Values are presented as a ratio of pAkt/Akt (mean ± SEM).

Statistical analysis

Statistical differences were assessed using two-tailed Student's t-tests for independent means (paired or unpaired as appropriate), one-way analysis of variance (ANOVA) followed by *post-hoc* Newman-Keuls tests for comparisons between multiple groups, or two-way ANOVA followed by *post-hoc* Bonferroni analysis to assess the effects of two independent variables. All statistical tests were carried out using GraphPad Prism software.

RESULTS

To assess the role of SIGIRR in mediating the response to TLR2 activation in the brain, mixed glial cells prepared from neonatal wildtype and SIGIRR$^{-/-}$ mice were incubated in the presence or absence of the TLR2 agonist, Pam_3CysSK_4 (Pam_3Cys_4) for 24h. Pam_3Cys_4 increased supernatant concentrations of pro-inflammatory cytokines TNFα and IL-6 in glia prepared from wildtype mice, which were augmented with cells from SIGIRR$^{-/-}$ mice with a significant genotype x treatment interaction ($F(1,20)=159.5$, $p<0.001$ and $F(1,20)=459.2$, $p<0.001$, 2-way ANOVA; Figure 1a,b, respectively). Similarly, when glia were incubated in the presence of Aβ for 24h, cells prepared from SIGIRR$^{-/-}$ mice responded more robustly than those from wildtype mice, and a significant treatment x genotype interaction was observed (TNFα: $F(1,20)=60.94$, $p<0.001$; IL-6: $F(1,20)=29.86$, $p<0.001$, 2-way ANOVA; Figure 1c,d, respectively). Previous reports have suggested that enhanced TLR4 and IL-1RI expression may account for the pro-inflammatory phenotype identified in adult SIGIRR-deficient animals (Costello et al., 2011b; Watson et al., 2010). To further investigate the enhanced response to TLR2-activating stimuli, expression of TLR2 mRNA was assessed in glia from wildtype and SIGIRR$^{-/-}$ mice. Unlike our previous findings on TLR4 expression, resting levels of TLR2 mRNA were not altered in glia from SIGIRR$^{-/-}$ animals (Figure 1e,f). However exposure to both Pam_3Cys_4 and Aβ enhanced TLR2 expression, and this effect was exaggerated in glia from SIGIRR$^{-/-}$ animals (interaction: $F(1,19)=13.77$, $p<0.01$ and $F(1,19)=34.63$, $p<0.001$, 2-way ANOVA; Figure 1e,f, respectively).

To investigate the differential effect of Aβ on microglia and astrocytes, enriched cultures of both were exposed to Aβ for 6h. Aβ increased expression of TNFα mRNA in microglia, which was more profound than that seen in astrocytes, with a significant cell-type x treatment interaction ($F(1,8)=16.63$, $p<0.01$, 2-way ANOVA; Figure 2a). Basal levels of IL-6

were significantly lower in microglia than astrocytes (**p<0.01, 2-way ANOVA), although Aβ enhanced its expression in both cell types with significant interaction between groups ($F(1,7)=8.74$, $p<0.05$, 2-way ANOVA; Figure 2b). To determine the contribution of TLR2 and SIGIRR to Aβ-mediated inflammatory changes, their mRNA expression was assessed in astrocytes and microglia following 6h exposure to Aβ. Both TLR2 and SIGIRR were expressed to a greater degree in microglia compared with astrocytes ($F(1,8)=110.3$, ***p<0.001, $F(1,8)=84.13$, ***p<0.001, 2-way ANOVA; Figure 2c,d respectively). While Aβ further enhanced the expression of TLR2 in microglia ($F(1,8)=23.46$, **p<0.01, 2-way ANOVA; Figure 2c), the expression of SIGIRR was significantly reduced ($F(1,8)=10.24$, **p<0.01, 2-way ANOVA; Figure 2d). Interestingly, neither was significantly altered in astrocytes in response to Aβ (Figure 2c,d). Our findings have highlighted microglia as the primary glial cell responsible for the expression of TLR2 and SIGIRR, along with their TLR2-dependent modulation by Aβ. To confirm the role of TLR2 activation in this modulation, microglia were incubated with Pam_3Cys_4 for 6h. These cells similarly displayed increased expression of TNFα and IL-6 (*p<0.05, Student's t-test; Figure 3a,b respectively). Activation of TLR2 with Pam_3Cys_4 also mimicked the effects of Aβ by increasing the expression of TLR2 (**p<0.01, Student's t-test; Figure 3c) while significantly reducing SIGIRR mRNA (*p<0.05, Student's t-test; Figure 3d).

The physiological consequences of Aβ-mediated inflammation in the brain have been widely documented. In particular we, among others, have illustrated the detrimental effects of acute Aβ application on hippocampal synaptic plasticity (Costello et al., 2005; Lyons et al., 2012). To address the potential involvement of TLR2 in mediating Aβ-induced synaptic dysfunction, LTP was recorded in hippocampal slices from naive wildtype and TLR2-deficient mice, in the presence or absence of $Aβ_{[1-40]}$ (200nM). Application of Aβ to the perfusion media (40 min prior to LTP induction) significantly impaired LTP in slices from wildtype animals

(n=4, Figure 4a,c), however no Aβ-induced alteration was observed in slices from $TLR2^{-/-}$ mice (interaction: $F(1,11)=6.3$, $p<0.05$, 2-way ANOVA; n=3-4, Figure 4b,c). To determine the role of TLR2 in Aβ-induced cytokine production, microglia were exposed to Aβ in the presence or absence of an αTLR2 antibody, to block TLR2 activation. A significant interaction between treatments identified that the presence of αTLR2 prevented the Aβ-induced expression and release of TNFα from microglia ($F(1,32)=36.88$, $F(1,32)=34.27$, $p<0.001$, 2-way ANOVA; Figure 5a,b respectively). Similarly, αTLR2 attenuated the change in IL-6 expression in response to Aβ (interaction: $F(1,32)=5.66$, $p<0.05$, 2-way ANOVA; Figure 5c) and prevented the Aβ-induced increase of IL-6 supernatant concentration ($F(1,8)=30.74$, $^{++}p<0.01$, compared to IgG+Aβ, 2-way ANOVA; Figure 5d). Taken together, these findings confirm that TLR2 mediates the inflammatory effects of Aβ which we have observed in microglial cells.

To investigate the inverse relationship between expression of TLR2 and SIGIRR, data obtained in the presence or absence of Aβ was used to illustrate a significant anti-correlation in their expression ($F(1,7)=28.87$, $p<0.001$; Figure 6a). Furthermore, we established that the Aβ-induced modulation in the expression of TLR2 and SIGIRR could be rectified by application of αTLR2 ($F(1,32)=31.26$, $^{+++}p<0.01$, $F(1,32)=14.52$, $^{+++}p<0.001$, 2-way ANOVA; Figure 6b,c, respectively). Interestingly however, incubation of microglia with αTLR2 alone significantly reduced the expression of TLR2 mRNA ($F(1,32)=90.91$, $^{***}p<0.001$, 2-way ANOVA; Figure 6b) which, unexpectedly, was associated with an increase in SIGIRR ($F(1,32)=37.03$, $^{**}p<0.01$, 2-way ANOVA; Figure 6c). Hippocampal slices from C57BL/6 mice were exposed to $A\beta_{[1-40]}$, following incubation with αTLR2 or control IgG. Stable and robust LTP was recorded in slices treated with either IgG or αTLR2 alone (n=3, Figure 7a,c). However, while Aβ significantly impaired LTP in IgG-treated slices ($F(1,9)=6.29$, $^{*}p<0.05$, 2-way ANOVA; n=4), a significant interaction revealed that this was prevented by prior

incubation with αTLR2 ($F(1,9)=6.52$, $p<0.05$, 2-way ANOVA; n=3; Figure 7b,c). In addition, hippocampal slices incubated with αTLR2 for 1h revealed significantly higher expression of SIGIRR mRNA, compared with IgG-treated controls (**$p<0.01$, Student's t-test; n=4; Figure 7d), an effect which was not identified in hippocampal tissue or microglia isolated from $TLR2^{-/-}$ mice compared with their wildtype counterparts (data not shown).

In light of the interesting observation that αTLR2 augments SIGIRR mRNA expression in both microglia and hippocampal tissue, we attempted to address the potential mechanism which may underlie its transcription. Relatively little information exists regarding the regulation of SIGIRR expression, which is known to be cell-, location- and stimulus-specific (Riva et al., 2012). ChIP analysis (http://www.sabiosciences.com/chipqpcrsearch.php) suggested that PPARγ is a transcription factor for SIGIRR. We first determined whether αTLR2-induced SIGIRR expression may be an effect of Fcγ receptor ligation by pre-incubating microglia in the presence or absence of an αCD16/CD32 blocking antibody (10μg/ml, 90min). A two-way ANOVA revealed a significant effect of αTLR2 ($p<0.01$), with SIGIRR expression significantly enhanced (2.47±0.23 RQ) compared with cells exposed to control IgG (1.46±0.24 RQ, $p<0.05$), following Fcγ receptor blockade. We next assessed the involvement of PPARγ activation and accordingly, in the presence of the PPARγ antagonist GW9662 (GW), the αTLR2-induced increase in SIGIRR expression was prevented (interaction: $F(1,28)=4.96$, $p<0.05$, 2-way ANOVA; Figure 8a). As the anti-inflammatory effects of PPARγ are known to be associated with Akt (Paintlia et al., 2006; Xing et al., 2008), we assessed activation of Akt in microglia following αTLR2 application and identified a significant increase in Akt phosphorylation following 4-6h incubation with αTLR2 ($F(2,6)=7.26$, *$p<0.05$, 1-way ANOVA; Figure 8b,c). Furthermore, a significant interaction revealed that the αTLR2-induced increase in SIGIRR was prevented by the PI3 kinase inhibitor

LY294002 (LY; $F(1,28)=4.64$, $p<0.05$, 2-way ANOVA; Figure 8d). Based on these findings, we can propose that αTLR2 enhances expression of SIGIRR through a mechanism which is likely to involve activation of PI3kinase/Akt and PPARγ.

DISCUSSION

The significant finding in this study is that anti-TLR2 antibody increases the expression of SIGIRR, which acts as a negative regulator of TLR2-mediated signalling and thereby inhibits Aβ-induced cytokine production and synaptic dysfunction.

Results obtained in glial cells from SIGIRR-deficient animals demonstrate a clear role for SIGIRR as a negative regulator of TLR2-mediated responses in the brain. Specifically, the effect of Pam_3Cys_4 was enhanced in glia prepared from SIGIRR-deficient mice, mimicking the changes previously observed in response to LPS (Watson et al., 2010). In contrast to the changes previously identified in TLR4, SIGIRR-deficiency was not associated with a basal increase in expression of TLR2 (Costello et al., 2011b; Watson et al., 2010). Interestingly, auto-regulation of TLR2 in response to activation was exaggerated in glia from $SIGIRR^{-/-}$ mice. In a similar manner to Pam_3Cys_4, Aβ induced the release of cytokines from glia, to a greater extent in cells prepared from SIGIRR-deficient mice. This provides indirect support for the previous findings which indicate that TLR2 may mediate the effects of Aβ in glial cells (Jana et al., 2008; Reed-Geaghan et al., 2009). As expression of TLR2 was found to be greater in microglia than astrocytes, suggesting that microglia are the primary glial cell responsible for mediating the inflammatory effects of its activation, we examined the modulatory effect of an αTLR2 antibody on Aβ-induced changes in microglia. The Aβ-induced expression and release of TNFα and IL-6 were completely attenuated by the antibody, indicating a regulatory role for TLR2 in Aβ-induced cytokine production by microglia.

Hippocampal synaptic plasticity is regarded as a primary physiological target of inflammatory insult in the brain (Lynch, 2014b). Pro-inflammatory cytokines such as IL-1β (Vereker et al., 2000b), TNFα (Lyons et al., 2012) and interferon-γ (Kelly et al., 2013), as well as agonists of TLR2 and 4 (Costello et al., 2011a; Costello et al., 2011b; Vereker et al., 2000a), have been shown to be detrimental to LTP. Impairment of LTP has also been reported following acute exposure to Aβ (Costello and Herron, 2004; Costello et al., 2005; Lyons et al., 2012; Minogue et al., 2007). Here we illustrate a role for TLR2 in mediating this effect, as the Aβ-induced impairment in LTP was prevented in hippocampal slices exposed to αTLR2, and slices prepared from TLR2-deficient animals.

The modulatory effects of αTLR2 were accompanied by up-regulation of SIGIRR expression. SIGIRR was expressed to a greater extent in microglia than astrocytes, and found to be further increased in microglia and hippocampal tissue following incubation with αTLR2. Expression of SIGIRR negatively correlated with TLR2, as an Aβ-induced increase in TLR2 was associated with reduced SIGIRR. It is likely therefore that αTLR2 can compensate for the deleterious effects of Aβ by redressing the balance between TLR2 and SIGIRR. Indeed, the αTLR2-induced increase in SIGIRR suggests that the effects of the antibody are not confined to blocking TLR2 activation, since similar alterations are not seen in hippocampal tissue from $TLR2^{-/-}$ mice. As changes induced by TLR4 and IL-1R are also negatively regulated by SIGIRR, the potential for αTLR2 to impact upon TLR4- and IL-1R-induced signalling also exists. However, we have found that although αTLR2 antibody attenuated Aβ-induced release of TNFα and IL-6 from mixed glia, αTLR4 did not exert any modulatory effect. While down-regulation of SIGIRR has been widely reported in response to LPS, infection and immune challenge (Garlanda et al., 2004; Huang et al., 2006; Riva et al., 2012; Veliz Rodriguez et al., 2012; Wald et al., 2003), few factors have been reported to augment its expression. One study

reported an increase in SIGIRR mRNA in dendritic cells from Peyer's patches, following exposure to LPS (Davies et al., 2010). Increases have also been observed in porcine antigen presenting cells in response to Pam_3Cys_4 and the probiotic *L. jensenii* (Villena et al., 2012) and in cornea infected with *P. aeruginosa*, following treatment with vasoactive intestinal peptide (Jiang et al., 2012).

The mechanisms that control SIGIRR expression under either resting or inflammatory conditions are relatively unexplored; however ChiP analysis has identified multiple promotor regions for the transcription factor PPARγ in the *sigirr* gene. The anti-inflammatory effects of PPARγ activation in the brain, and particularly in response to Aβ, have been widely reported (Camacho et al., 2004; Costello et al., 2005; Minogue et al., 2007). We show that the PPARγ inhibitor, GW9662, attenuated the αTLR2-induced increase in SIGIRR, thus supporting a role for PPARγ activation in regulating SIGIRR expression. The protective effects of PPARγ are associated with activation of the PI3 kinase/Akt pathway in microglia (Xing et al., 2008), oligodendrocyte precursors (Paintlia et al., 2006) and cerebellar granule neurons (Uryu et al., 2002). We demonstrate that αTLR2 induces Akt phosphorylation and that the PI3 kinase inhibitor LY294002 prevented the αTLR2-induced modulation of SIGIRR.

CONCLUSIONS

We have identified SIGIRR as a negative regulator of TLR2-mediated cytokine production in the brain, and highlight an interaction between the receptors as a factor in setting the threshold for activation of microglia. We propose that a shift in the balance between these receptors contributes to the negative impact of Aβ on synaptic function, that this balance is altered by activation of TLR2, and that an antibody targeting TLR2 may offer therapeutic potential to Alzheimer's disease-related pathology.

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FIGURE LEGENDS

Figure 1: SIGIRR-deficiency exaggerates the inflammatory response to Pam_3Cys_4 and Aβ in glial cells

Exposure of glial cells from wildtype and $SIGIRR^{-/-}$ mice to Pam_3Cys_4 and Aβ (24h) induced release of TNFα (a,c) and IL-6 (b,d,), a significant genotype x treatment interaction revealed that this effect was further enhanced in cells prepared from $SIGIRR^{-/-}$ mice ($p<0.01$, 2-way ANOVA). Both Pam_3Cys_4 and Aβ significantly increased glial cell expression of TLR2 mRNA

(e,f respectively). A similar interaction revealed that TLR2 was increased to a significantly greater extent in cells from SIGIRR$^{-/-}$ animals compared with wildtypes (e; $p<0.01$, f; $p<0.001$, 2-way ANOVA). **$p<0.01$, ***$p<0.001$, compared to wildtype control; $^{+++}p<0.001$, compared to wildtype cells exposed to Pam_3Cys_4 or Aβ (2-way ANOVA).

Figure 2: TLR2 and SIGIRR are more abundant in microglia than astrocytes and their expression is differently modulated by Aβ

(a) Expression of TNFα mRNA was increased in microglia following 6h exposure to Aβ, and to a significantly greater extent than that seen in Aβ-treated astrocytes (cell-type x treatment interaction: $p<0.01$, 2-way ANOVA). ***$p<0.001$, compared to astrocyte controls; $^{+++}p<0.001$, compared to Aβ-treated astrocytes. (b) Basal expression of IL-6 mRNA was lower in microglia compared to astrocytes, although a significant increase was observed in both cell types in response to Aβ (cell-type x treatment interaction: $p<0.05$, 2-way ANOVA). **$p<0.01$, compared to astrocyte controls; $^{+++}p<0.001$, compared to Aβ-treated astrocytes. TLR2 (c) and SIGIRR (d) were both expressed to a greater extent in microglia compared with astrocytes (2-way ANOVA). Exposure to Aβ significantly increased TLR2 (c) and significantly reduced SIGIRR (d) in microglia compared with controls (**$p<0.01$, 2-way ANOVA), but did not alter expression of either in astrocytes. **$p<0.01$, ***$p<0.001$, compared to astrocyte controls; $^{++}p<0.01$,$^{+++}p<0.001$, compared to Aβ-treated astrocytes. Bars represent comparisons made between control and Aβ-treated microglia.

Figure 3: The inflammatory response to Pam_3Cys_4 is associated with altered expression of TLR2 and SIGIRR in microglia

Pam_3Cys_4 (6h) induced a significant increase in the expression of (a) TNFα and (b) IL-6 in microglia, compared to values obtained in un-treated cells. (c) TLR2 mRNA was significantly

enhanced in microglia following exposure to Pam_3Cys_4, compared to controls. (d) Microglial expression of SIGIRR was significantly reduced in response to Pam_3Cys_4 when compared with un-treated controls. *p<0.05, **p<0.01 (Student's t-test).

Figure 4: The Aβ-induced impairment in hippocampal LTP is prevented in TLR2-deficient mice

(a) LTP recorded in hippocampal slices from wildtype mice in the presence or absence of $A\beta_{[1-40]}$ (200nM; n=4 slices from 4 mice). (b) LTP recorded in hippocampal slices from $TLR2^{-/-}$ mice, in the presence or absence of Aβ (n=3-4 slices from 3-4 mice). (c) Mean %EPSP slope recorded 60 min following TBS application revealed a significant genotype x treatment interaction (p<0.05, 2-way ANOVA). ***p<0.001, compared to wildtype control (2-way ANOVA). Arrows indicate application of TBS. Insets illustrate representative EPSPs (average of 4 consecutive traces) recorded immediately prior to, and 60 min following TBS.

Figure 5: αTLR2 antibody inhibits the Aβ-induced expression and release of pro-inflammatory cytokines

The mRNA expression (a) and supernatant concentration (b) of TNFα were significantly increased following the application of Aβ (6h) compared with values obtained in the presence of normal IgG alone. The increase in expression (a) and release (b) of TNFα was inhibited when Aβ was applied in the presence of αTLR2, providing a significant interaction between treatments (p<0.001, 2-way ANOVA). Aβ significantly increased the mRNA expression (c) and supernatant concentration (d) of IL-6, compared with microglia exposed to normal IgG. Application of Aβ in the presence of αTLR2 prevented the increase in IL-6 expression (c) and release (d) compared to values obtained when Aβ was applied in the presence of an IgG control.

**p<0.01, ***p<0.001, compared to microglia exposed to IgG alone; $^{++}$p<0.01, $^{+++}$p<0.001, compared to microglia treated with IgG+Aβ (2-way ANOVA).

Figure 6: αTLR2 antibody reduces microglial expression of TLR2, enhances expression of SIGIRR, and prevents their modulation by Aβ

(a) Data obtained from microglia following exposure Aβ reveal a significant anti-correlation in the expression of TLR2 and SIGIRR (p<0.001). (b) Basal expression of TLR2 was significantly reduced in microglia treated with αTLR2 compared with cells exposed to IgG. The Aβ-induced increase in TLR2 was inhibited in cells exposed to αTLR2. (c) SIGIRR mRNA expression was significantly increased in microglia treated with αTLR2 compared with cells exposed to IgG, while the Aβ-induced reduction in SIGIRR was prevented in αTLR2-treated microglia. **p<0.01, ***p<0.001, compared to microglia exposed to IgG alone; $^{+++}$p<0.001, compared to cells treated with IgG+Aβ (2-way ANOVA).

Figure 7: αTLR2 antibody prevents the Aβ-induced impairment in hippocampal LTP, which is associated with increased SIGIRR expression

(a) LTP induced by TBS was similar in hippocampal slices pre-exposed to IgG and αTLR2 (1h). In the presence of Aβ, the LTP recorded in slices pre-treated with control IgG was significantly lower than values obtained in control slices pre-exposed to IgG alone (a,c) or αTLR2-treated slices in the presence of Aβ (b,c). (c) Mean %EPSP slope recorded 60 min following TBS application revealed a significant interaction between αTLR2 and Aβ treatments (p<0.05, 2-way ANOVA; n=3-4 slices from 3-4 mice). *p<0.05, compared to IgG-treated tissue; +p<0.05, compared to tissue exposed to IgG+Aβ (2-way ANOVA). Arrows indicate application of TBS. Insets illustrate representative EPSPs (average of 4 consecutive

traces) recorded immediately prior to, and 60 min following TBS. (d) SIGIRR expression was significantly greater in hippocampal tissue treated *ex vivo* with αTLR2 (1h), compared tissue exposed to control IgG (**p<0.01, Student's t-test).

Figure 8: The αTLR2-induced increase in SIGIRR expression is mediated by PI3 kinase/Akt and PPARγ

(a) Co-application of GW with αTLR2 significantly attenuated the increase in SIGIRR expression identified in the presence of αTLR2 alone (αTLR2 x Aβ interaction: p<0.05, 2-way ANOVA). **p<0.01, ***p<0.001, compared with cells exposed to IgG alone; $^{+++}$p<0.001, compared to cells treated with αTLR2 alone (2-way ANOVA). (b) Representative immunoreactive bands corresponding to pAkt, tAkt and β-actin, identified in untreated microglia and microglia exposed to either control IgG or αTLR2 (4h). (c) A significant increase in Akt phosphorylation was identified in αTLR2-treated microglia, compared with un-treated cells (*p<0.05, 1-way ANOVA). (d) αTLR2 significantly increased SIGIRR expression compared to IgG-treated microglia, which was prevented in the presence of LY (αTLR2 x Aβ interaction: p<0.05, 2-way ANOVA).

Figure 1

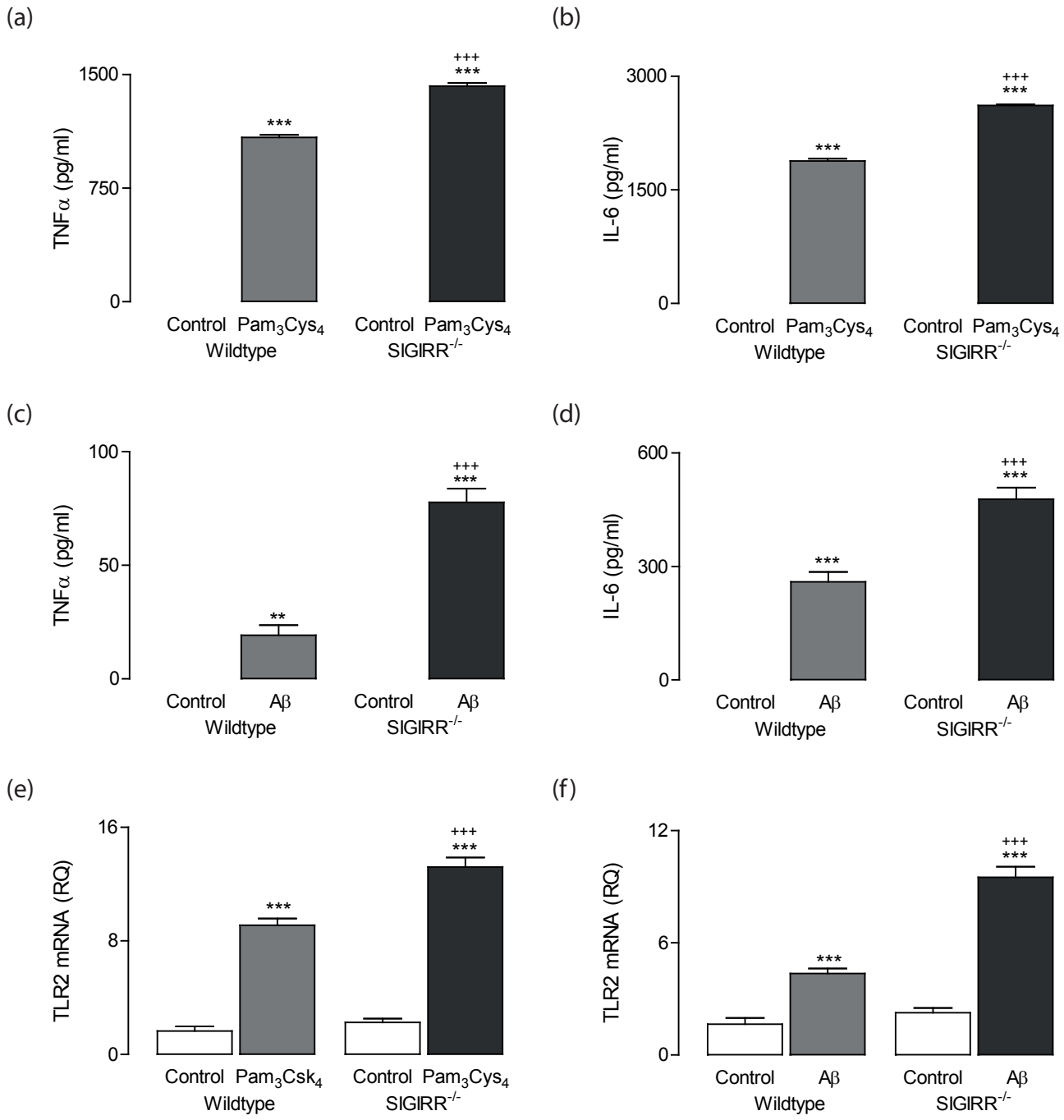
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Figure 2

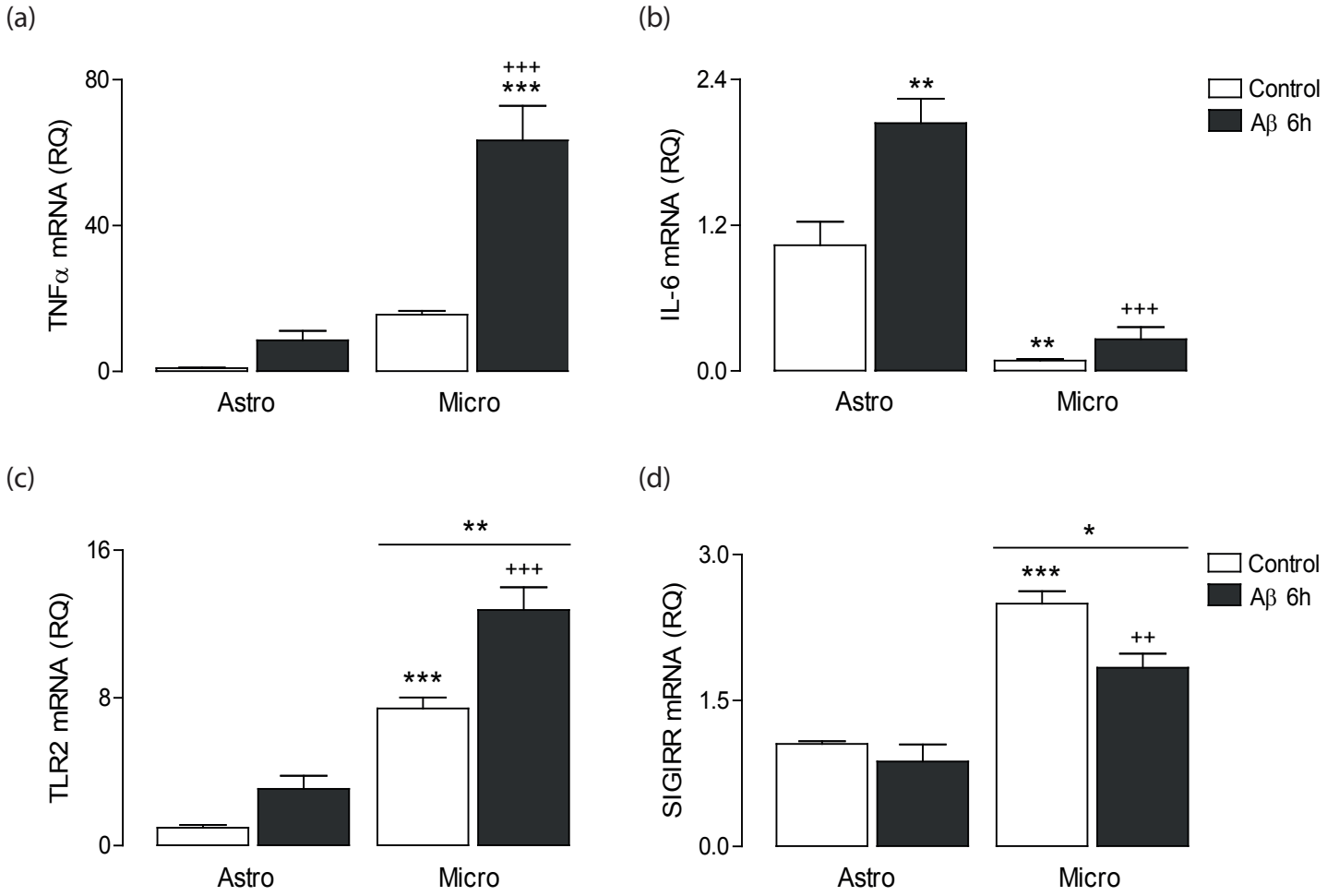
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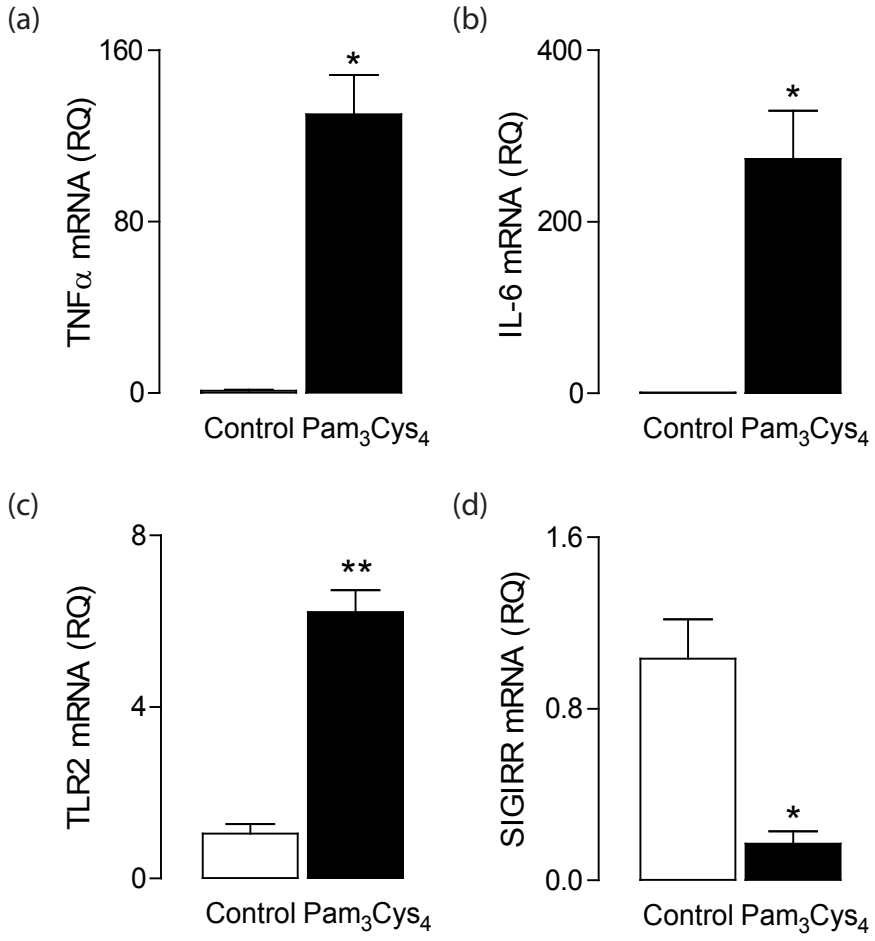
Figure 3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Pam3Cys4
(c)
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TLR2 mRNA (RQ)
**
Control
Pam3Cys4
(d)
1.6
0.8
0.0
SIGIRR mRNA (RQ)
*
Control
Pam3Cys4

Figure 4

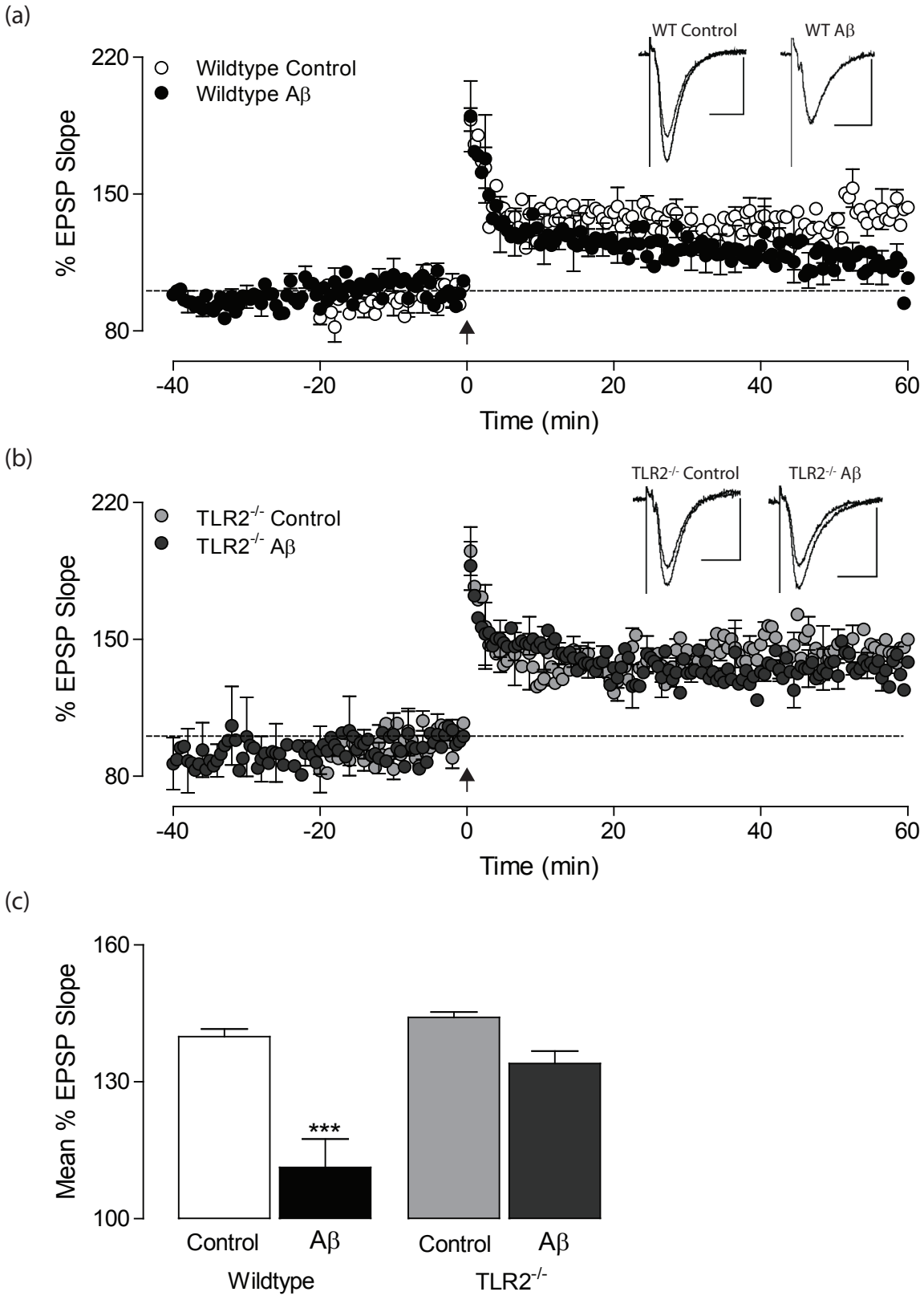

(a)
WT Control
WT Aβ
Wildtype Control
Wildtype Aβ
% EPSP Slope
220
150
80
-40
-20
0
20
40
60
Time (min)
(b)
TLR2-/- Control
TLR2-/- Aβ
TLR2-/- Control
TLR2-/- Aβ
% EPSP Slope
220
150
80
-40
-20
0
20
40
60
Time (min)
(c)
Mean % EPSP Slope
160
130
100
***
Control
Aβ
Control
Aβ
Wildtype
TLR2-/-

Figure 5

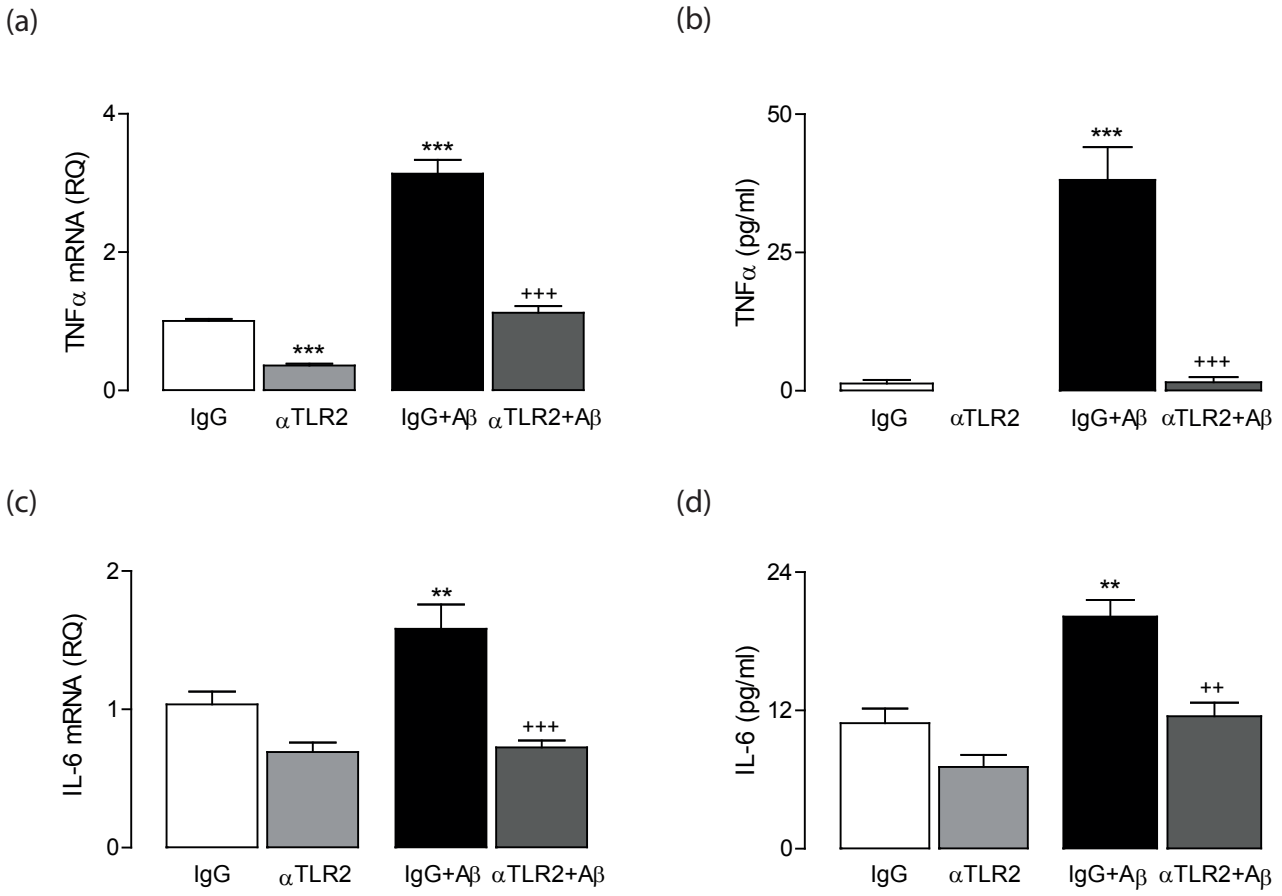

(a)
TNFα mRNA (RQ)
4
2
0
***
***
+++
IgG
αTLR2
IgG+Aβ
αTLR2+Aβ
(b)
TNFα (pg/ml)
50
25
0
***
+++
IgG
αTLR2
IgG+Aβ
αTLR2+Aβ
(c)
IL-6 mRNA (RQ)
2
1
0
**
+++
IgG
αTLR2
IgG+Aβ
αTLR2+Aβ
(d)
IL-6 (pg/ml)
24
12
0
**
++
IgG
αTLR2
IgG+Aβ
αTLR2+Aβ

Figure 6

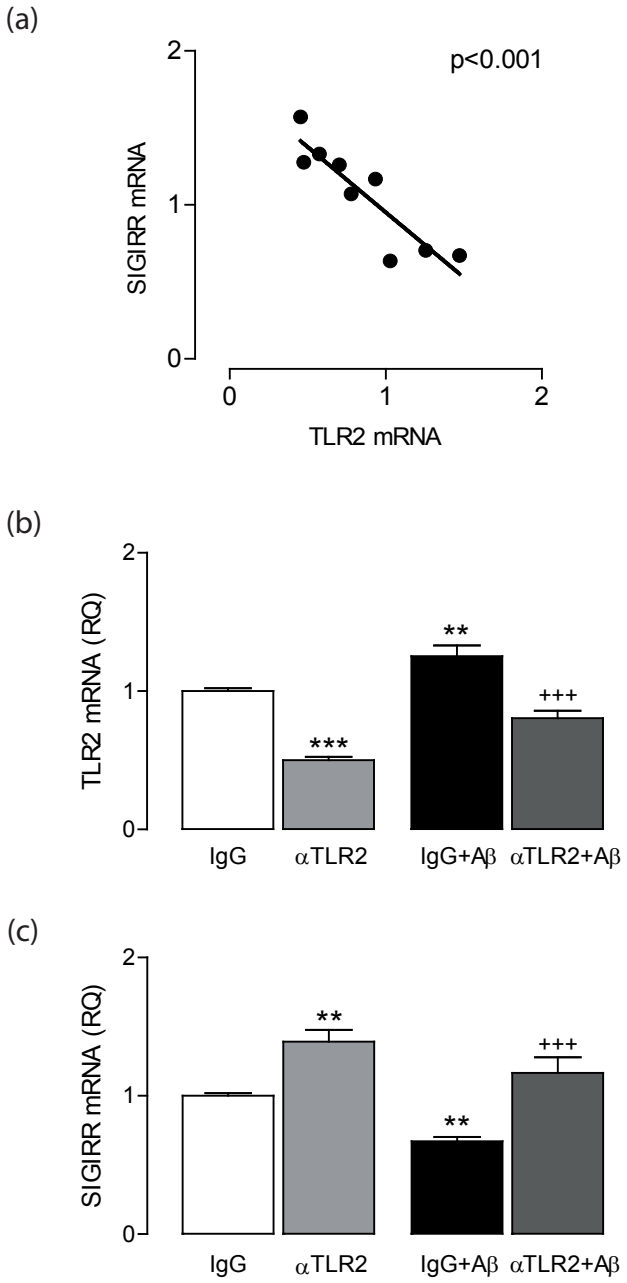

(a)
p<0.001
SIGIRR mRNA
TLR2 mRNA
0
1
2
(b)
TLR2 mRNA (RQ)
**
***
+++
IgG
αTLR2
IgG+Aβ
αTLR2+Aβ
(c)
SIGIRR mRNA (RQ)
**
**
+++
IgG
αTLR2
IgG+Aβ
αTLR2+Aβ

Figure 7

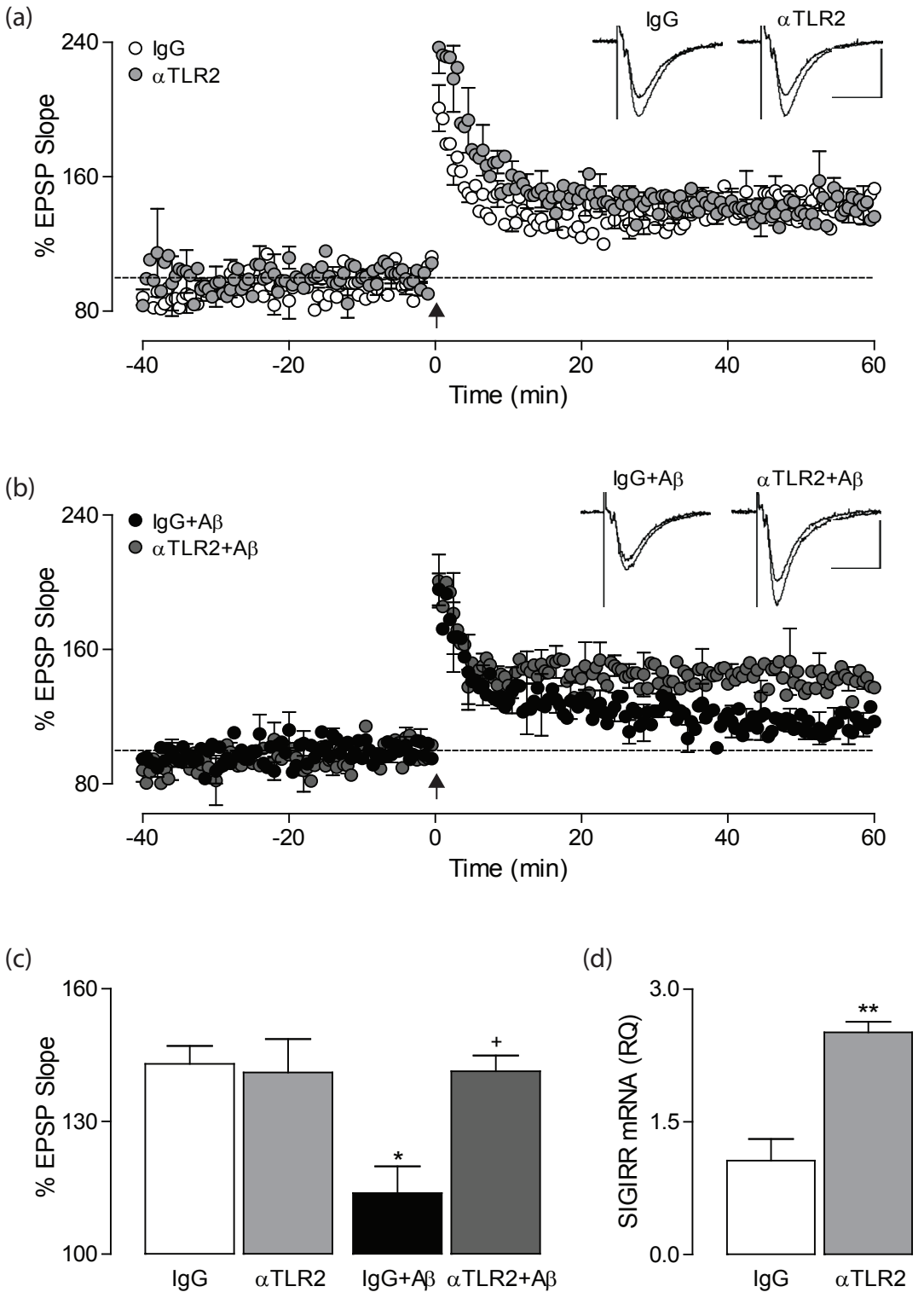
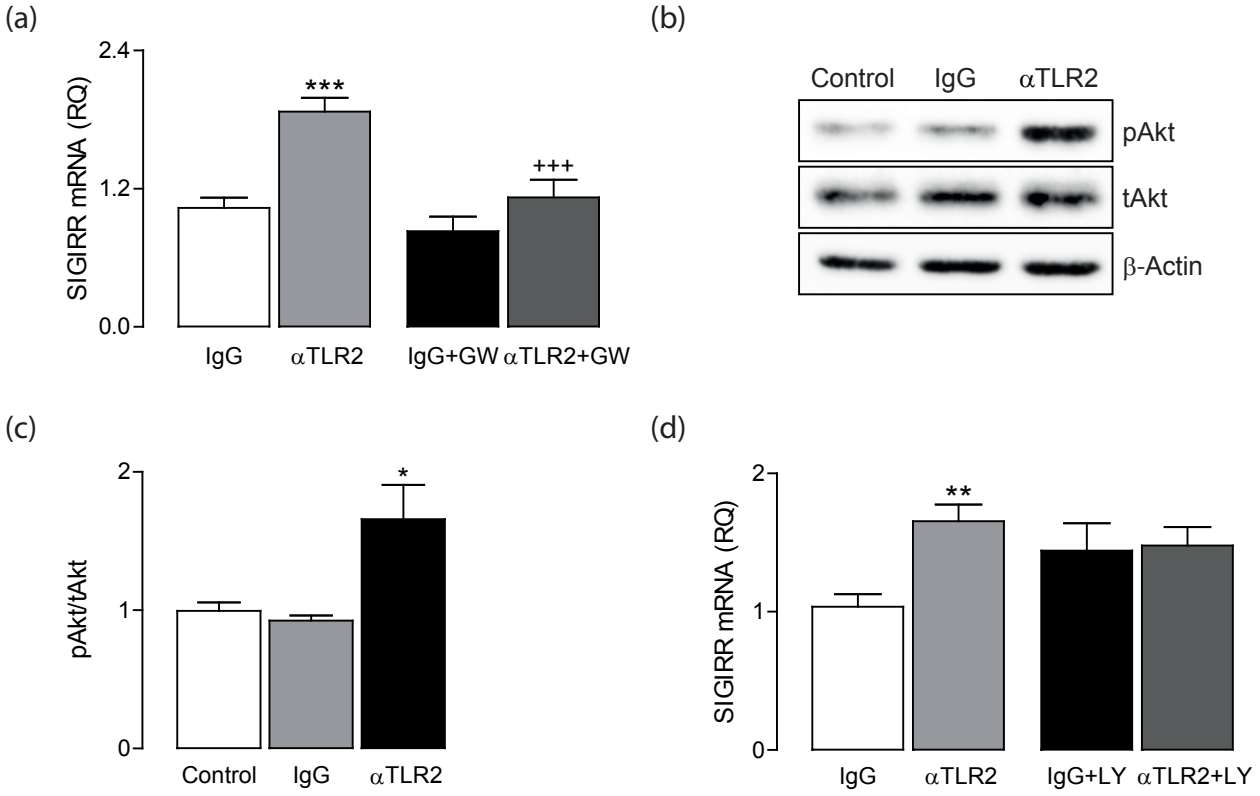

(a)
IgG
αTLR2
% EPSP Slope
240
160
80
-40
-20
0
20
40
60
Time (min)
(b)
IgG+Aβ
αTLR2+Aβ
(c)
160
130
100
*
+
IgG
αTLR2
IgG+Aβ
αTLR2+Aβ
(d)
SIGIRR mRNA (RQ)
3.0
1.5
0.0
**

Figure 8


(a)
SIGIRR mRNA (RQ)
2.4
1.2
0.0
***
+++
IgG
αTLR2
IgG+GW
αTLR2+GW
(b)
Control
IgG
αTLR2
pAkt
tAkt
β-Actin
(c)
pAkt/tAkt
2
1
0
*
Control
IgG
αTLR2
(d)
SIGIRR mRNA (RQ)
2
1
0
**
IgG
αTLR2
IgG+LY
αTLR2+LY