# SIGIRR modulates the inflammatory response in the brain

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**ABSTRACT**

One of the more recently described members of the interleukin-1 (IL-1) receptor family, single-Ig-Interleukin-1 related receptor (SIGIRR), has been identified as a negative regulator of inflammation in several tissues. It modulates the responses triggered by stimulation of Toll-like receptor (TLR) 4 and IL-1 in several peripheral cell types, possibly in an NFκB-dependent manner. Consistently, responses to lipopolysaccharide (LPS) are exaggerated in SIGIRR-deficient mice and the symptoms of experimental inflammatory conditions are more profound in these animals. Here we set out to establish whether the absence of SIGIRR was associated with inflammatory changes in the brain and report that, LPS induced a greater effect on CD40 and ICAM mRNA in mixed glia prepared from SIGIRR$^{-/-}$, compared with wildtype mice. This was associated with parallel changes in TNFα and IL-6 at mRNA and protein levels, an effect which was observed in purified microglia but not astrocytes. Similarly, LPS exerted a more profound effect on microglial activation and cytokine production in hippocampal tissue prepared from SIGIRR$^{-/-}$, compared with wildtype mice. The effect of LPS on exploratory behaviour was also accentuated in SIGIRR$^{-/-}$ mice. The evidence suggests that these changes are a likely consequence of increased hippocampal expression of CD14 and TLR4, and NFκB activation in SIGIRR$^{-/-}$ mice.

## 1. Introduction

Single-Ig-Interleukin-1 related receptor (SIGIRR) is a structurally distinct member of the TLR/IL-1R family (Thomassen et al., 1999). It is present in most tissues, with highest expression in kidney and gastrointestinal tract (Wald et al., 2003), notably in epithelial cells (Polentarutti et al., 2003). SIGIRR is also present in brain (Costelloe et al., 2008; Polentarutti et al., 2003) where it is expressed on neurons, microglia and astrocytes (Andre et al., 2005).

SIGIRR is unable to bind IL-1α, IL-1β or IL-1ra (Thomassen et al., 1999) but it can interact with IRAK and TRAF-6, which likely infers its ability to inhibit TLR/IL-1-induced signalling (Qin et al., 2005b; Wald et al., 2003). Its inability to respond to IL-1R-triggered signalling stems from the fact that it lacks two essential amino acids in its highly-conserved TIR domain; therefore cells in which various SIGIRR constructs were expressed failed to exhibit evidence of NFκB activation (Polentarutti et al., 2003; Thomassen et al., 1999). It has been suggested that the single extracellular Ig domain is too short to fold around IL-1-like ligands (Thomassen et al., 1999), and in the absence of any data suggesting possible binding of these ligands to the receptor, SIGIRR remains an orphan receptor. However, we have shown that the effects of IL-1F5 are absent in mixed glia prepared from SIGIRR$^{-/-}$ mice suggesting that the anti-inflammatory effects of this cytokine are mediated, at least in part, by SIGIRR (Costelloe et al., 2008).

SIGIRR is a negative regulator of inflammation and so LPS-induced inflammatory responses in the intestine are exaggerated in SIGIRR$^{-/-}$ mice, while these mice also have a reduced threshold to lethal LPS challenge (Garlanda et al., 2004; Wald et al., 2003).

Similarly, kidney cells and tubular epithelial cells (Lech et al., 2007; Qin et al., 2005b), splenocytes (Wald et al., 2003), bone-derived macrophages and dendritic cells (Garlanda et al., 2007; Garlanda et al., 2004) prepared from SIGIRR$^{-/-}$ mice respond more robustly to LPS and/or IL-1 than cells prepared from wildtype mice, generally assessed in terms of production of chemokines and inflammatory cytokines. Further evidence of the anti-inflammatory influence of SIGIRR has been obtained from studies in which the receptor was overexpressed; for example, Huang and colleagues demonstrated that RAW cells transfected with SIGIRR resulted in a decreased production of inflammatory cytokines, IL-1β, IL-12, IL-18 and IFN-γ (Huang et al., 2006). In addition to a role of SIGIRR in attenuating Toll-like receptor (TLR)4 and IL-IRI signalling, the evidence suggests that SIGIRR also attenuates inflammatory changes induced by activation of TLR1, TLR3 and TLR9 by lipopeptide (Pam3Cys), polyinosinic:polycytidylic acid (Poly I:C) and CpG-DNA, respectively (Garlanda et al., 2004; Lech et al., 2007; Qin et al., 2005b; Wald et al., 2003; Xiao et al., 2007).

We set out to investigate whether the anti-inflammatory effects of SIGIRR extend to the brain and report that, *in vitro* and *in vivo*, the effects of LPS were exacerbated in SIGIRR$^{-/-}$ mice. Specifically we show that LPS-induced microglial activation and production of TNFα and IL-6 were enhanced in cultured cells or hippocampal tissue prepared from SIGIRR$^{-/-}$ mice, probably as a result of increased expression of CD14 and TLR4, and that these changes were accompanied by evidence of changes in exploratory behaviour.

## 2. Materials and methods

### *2.1. Animals*

Male C57BL/6 mice (2-4 or 10-12 months; Harlan UK) and male SIGIRR$^{-/-}$/TIR8$^{-/-}$ mice (a gift from Professor A Mantovani, Istituto Clinico Humanitas IRCCS, Milan; Garlanda et al., 2004) were maintained in the Bioresources Unit, Trinity College Dublin. All experiments were performed under licence from the Department of Health and Children (Ireland) and with local ethical approval. In one series of experiments, 2-4 month old mice were injected intraperitoneally with saline (200µl) or LPS (200µl; 10µg/mouse; *E. coli* serotype 0111.B4; Sigma Aldrich, UK) and killed by decapitation 3 hours later, and in a second experiment, young and aged mice (2-4 and 10-12 months respectively) were killed by decapitation. In both cases, hippocampal tissue was taken and stored for later analysis of markers of microglial activation and cytokine production (Lyons et al., 2009).

### *2.2. Analysis of behaviour*

One group of mice were injected intraperitoneally with saline (200µl) or LPS (200µl; 10µg/mouse; *E. coli* serotype 0111.B4; Alexis Biochemicals, UK) and assessed for exploratory and anxiety-related behaviour 30 minutes later. This timepoint was chosen for behavioural analysis because earlier experiments indicated that LPS-treated SIGIRR$^{-/-}$ mice showed no interest in exploring their environment 3 hours after injection. Mice were released into the same side of the outer corner of a square (60cm width×60cm length×35cm height) open field arena for 2 minutes and activity was recorded using a video camera and

advanced motion-recognition software package (Mediacruise Software, Canopus Corporation, UK). The arena was partitioned into 25 equal-sized squares (16 border and 8 central squares) with a circular cut-out in each of the central corner squares positioned around a central square. The time spent in the border and central squares was assessed, as was head-dipping and rearing behaviour.

*2.3. Preparation of cultured cells*

Mixed glia and neurons were prepared from cortices of wildtype and SIGIRR$^{-/-}$ mice as previously described (Downer et al., 2010). Briefly, to prepare mixed glia, mice were decapitated and brain tissue was placed in 2 ml Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Ireland) supplemented with 10% foetal bovine serum (Gibco, UK), penicillin (100 U/ml; Gibco, UK) and streptomycin (100 U/ml; Gibco, UK). Samples were triturated, passed through a sterile nylon mesh filter, centrifuged (2000g, 3 minutes, 20°C) and the pellets resuspended in DMEM. Cells were plated ($1x10^5$ cells/ml) onto 10-mm diameter coverslips coated with poly-l-lysine (Sigma, UK) in 24 well plates, grown at 37°C in a humidified environment (5% $CO_2$:95% air) for 10 days prior to treatment and medium was replaced every 3 days. After 2 weeks, mixed glia were incubated in the presence or absence of LPS (100 ng/ml; Sigma, UK) for 24 hours.

To prepare purified microglia and astrocytes, cells were grown in T25 flasks in DMEM as above. After 12 days the flasks were shaken for 2 hours at 110 rpm at room temperature and tapped several times to remove the non-adherent microglia. The supernatant was removed and centrifuged at 2,000 rpm for 5 minutes at 20ºC and the pellet was resuspended in 1 ml DMEM and cells were counted. Cells were pipetted onto poly-L-lysine-coated (60

μg/ml) coverslips in 24 well plates at a density of 0.5 x $10^5$ cells/ml and incubated for 2 hours before addition of warmed DMEM.

To prepare astrocytes, the flasks containing the adherent astrocytes were washed with sterile PBS and 1 ml of 0.05% w/v trypsin-EDTA was added at 37°C until the cells just began to detach. DMEM was added to the flask to inhibit the trypsin, the cells were spun down at 900 x g for 3 minutes, the pellet was resuspended in DMEM and cells were plated in 6 well plates at a density of 0.5 x $10^5$ cells/ml. Microglia and astrocytes were incubated with LPS in the same manner as mixed glia.

*2.4. Real-time PCR*

cDNA synthesis was performed on 1-2 μg total RNA prepared from hippocampal homogenates, using a high capacity cDNA RT kit (Applied Biosystems, US). Real-time PCR primers were delivered as "Taqman® Gene Expression Assays" containing forward and reverse primers, and a FAM-labeled MGB Taqman probe for each gene (Applied Biosystems, US). Primers used were as follows: IL-6 (Mm00446191_m1), TNF-α (Mm0043258_m1), CD40 (Mm0041895_m1), ICAM (Mm00516023_m1), TLR4 (Mm00445273_m1), CD14 (Mm00438094_g1), MCP-1 (Mm00441242_m1), IP-10 (Mm00445235_m1 and β-actin (Mm00607939_s1). A 1:4 dilution of cDNA was prepared and real-time PCR performed using Applied Biosystems 7300 Real-time PCR System. cDNA was mixed with qPCR™ Mastermix Plus (Applied Biosystems, US) and the respective gene assay. Mouse β-actin was used as an endogenous control and expression was conducted using a gene expression assay containing forward and reverse primers and a VIC-labeled MGB Taqman probe (#4352340E; Applied Biosystems, US).

Forty to sixty cycles were run as follows: 10 minutes at 95 °C and for each cycle, 15 s at 95 °C and 1 minute at 60 °C. Gene expression was calculated relative to the endogenous control and analysis was performed using the $2^{-\Delta\Delta CT}$ method.

*2.4. Analysis of cytokine concentrations*

IL-6 and TNF-α concentrations were analyzed by ELISA in hippocampal homogenates and in supernatants from cultured cells (Lyons et al., 2007). Hippocampal homogenates were equalized for protein concentrations (Bradford, 1976) and samples (100 µl) of this homogenate and of supernatant prepared from cultured cells, as well as standards, were added to 96-well plates coated with antibody (rat anti-mouse IL-6 or TNF-α antibody; 100 µl; 0.8 µg/ml for TNF-α and 1 µg/ml for IL-6). Triplicate samples and standards (100 µl; 0-2000pg/ml recombinant mouse TNF-α, or 0-5000pg/ml recombinant mouse IL-6) were added, and incubated with assay diluent (300 µl; PBS containing 10% FBS) for 2 hours (supernatants) or overnight (tissue homogenate). Samples were then washed and incubated for 2 hours in the presence of detection antibody (100 µl; 150 ng/ml biotinylated rat anti-mouse TNF-α or 1 µg/ml biotinylated rat anti-mouse IL-6 diluted in 1% BSA). Detection reagent (100 µl; HRP conjugated streptavidin; 1:250 dilution in assay diluent) was added, incubation continued for 20 minutes, samples were washed and substrate solution (100 µl; 1:1 mixture of $H_2O_2$ and tetramethylbenzidine) was added. Samples were incubated in the dark for 20 to 30 minutes and the reaction was stopped using 50 µl 1M $H_2SO_4$. Plates were read at 450 nm and cytokine concentrations were estimated from the appropriate standard curve and expressed as pg/mg protein for homogenate and pg/ml for supernatants.

*2.5. Western blotting*

Hippocampal tissue was homogenised in lysis buffer (composition in mM: Tris-HCl 10, NaCl 50, $Na_4P_20_7.H_2O$ 10, NaF 50, 1% Igepal, phosphatase inhibitor cocktail I and II, Protease Inhibitor cocktail; Sigma, UK). Samples were added to 2x SDS sample buffer (composition: Tris-HCl 100mM, pH 6.8, 4% SDS, 2% bromophenol blue, 20% glycerol; Sigma, UK) and heated at 70°C for 10 min. Samples (20μg) were separated on 10 or 12% standard SDS gels. Proteins were transferred to nitrocellulose membrane (Schleicher and Schuell, Germany) and blocked for 1 hour in Tris-buffered-saline-0.05% Tween 20 (TBS-T) and 5% non-fat dried milk at room temperature. Membranes were incubated overnight at 4ºC with anti-p-IκBα (ser32) (1:1000) or anti-p-IKKα/β (1:1000; Cell Signaling, US), anti-CD86 (1:200) or anti-CD80 (1:500; Santa Cruz Biotechnology, US) antibody in 5% Bovine Serum Albumin (BSA)/TBS-T, washed, and incubated with a peroxidase-conjugated secondary antibody anti-rabbit (1:2500; Sigma, UK) or peroxidase-conjugated secondary antibody anti-rat (1:1000; Sigma, UK) in 5% BSA/TBS-T for 1 hour. Immunoreactive bands were detected using enhanced chemiluminescence (Amersham Biosciences, UK), and blots were stripped (Re-blot Plus; Chemicon, US) and reprobed using anti-β-actin (1:10,000; Sigma, UK) in 2% non-fat dried milk/TBS-T and a peroxidase-conjugated secondary antibody anti-mouse (1:5000; Sigma, UK) in 2% non-fat dried milk/TBS-T. Images were captured using the Fujifilm LAS-3000 system.

*2.6. Histology*

Cryostat sections (10 μm) were prepared and assessed for MHCII expression as previously described (Lyons et al., 2007). Sections were mounted in aqueous mountant (Vectashield; Vector, UK) and viewed with a Zeiss 510 Meta confocal laser microscope with an Axiovert 200M inverted microscope. Hoescht (DAPI) staining of nuclei was visualized using the 543 nm helium neon laser. Negative control experiments were performed by replacing the primary antibody with normal IgG antibodies and using equal gain settings during acquisition and analysis.

*2.7. Statistical analysis*

Data were assessed using a two-tailed Student's t-test for independent means or a one- or two-way analysis of variance (ANOVA) followed by *post hoc* comparisons, using Newman-Keuls test to determine which conditions were significantly different from each other. Prism 4 software (GraphPad, US) was used to carry out statistical comparisons.

## 3. Results

*3.1 LPS induces a greater effect in glia prepared from SIGIRR$^{-/-}$ compared with wildtype mice*

SIGIRR has been identified as a negative regulator of TLR-mediated innate-immunity in several peripheral cells (Garlanda et al., 2007; Garlanda et al., 2004; Qin et al., 2005b; Wald et al., 2003). As LPS is widely known to induce a potent and robust neuroinflammatory response, we hypothesized that it would exert a greater effect in cells prepared from SIGIRR$^{-/-}$, compared with wildtype mice. To assess this, the responses of

mixed glial cells prepared from SIGIRR$^{-/-}$ and wildtype mice to LPS were compared in terms of cell surface markers of microglial activation and proinflammatory cytokine production. SIGIRR negatively regulates TLR-mediated signalling through inhibition of IRAK and TRAF-6 (Qin et al., 2005b; Wald et al., 2003). Therefore, we chose to evaluate CD40 since CD40-mediated signaling requires recruitment of TRAF-6 (Ishida et al., 1996). Additionally, we evaluated ICAM because CD40-CD40L interactions are known to up-regulate ICAM-1 expression (Saito et al., 2007). We report that LPS significantly increased CD40 and ICAM mRNA in mixed glial cells prepared from wildtype mice (*$p < 0.05$; ***$p < 0.001$; Newman-Keuls; Fig. 1a and b) but that the LPS-induced change was significantly greater in cell prepared from SIGIRR$^{-/-}$ mice ($^{+++}p < 0.001$ versus LPS-treated wildtype mice).

In parallel with the changes in CD40 and ICAM, while LPS triggered a significant increase in supernatant concentrations of IL-6 and TNFα obtained from cells prepared from wildtype mice (***$p < 0.001$; Newman-Keuls; Fig. 2a and b), the effect was significantly greater in cells prepared from SIGIRR$^{-/-}$ mice ($^{++}p < 0.01$; $^{+++}p < 0.001$ versus LPS-treated wildtype mice). The findings that mixed glial cells prepared from SIGIRR$^{-/-}$ mice were hyper-responsive to LPS are similar to those described by others in peripheral immune cells (Wald et al., 2003), and indicate that SIGIRR is also involved in dampening the immediate signaling events initiated by TLR4 activation. Having demonstrated the effect of LPS on mixed glial cultures, we prepared purified microglia and astrocytes from wildtype and SIGIRR$^{-/-}$ mice in an effort to identify the cell type primarily responsible for the observed effect. LPS increased supernatant concentrations of IL-6 and TNFα in both astrocytes and microglia obtained from wildtype mice (***$p < 0.001$; Newman-Keuls; Fig. 2d-f) and this

effect was significantly enhanced in microglia, but not astrocytes, prepared from SIGIRR$^{-/-}$ mice ($^{+++}p < 0.001$; Newman-Keuls; Fig. 2d and f; LPS treatment in wildtype mice versus SIGIRR$^{-/-}$ mice).

*3.2. Intraperitoneal administration of LPS increases markers of microglial activation in hippocampus to a greater extent in SIGIRR$^{-/-}$ mice*

On the basis of these findings, we considered that a deficiency in SIGIRR, combined with an LPS challenge, would lead to increased inflammation in the brain. To assess this, wildtype and SIGIRR$^{-/-}$ mice were injected intraperitoneally with LPS and hippocampal tissue investigated for changes 3 hours later. In agreement with previous data obtained from rat, LPS significantly increased CD40 mRNA and ICAM mRNA (Downer et al., 2010) in hippocampus of wildtype mice (*$p < 0.05$; **$p < 0.01$; Newman-Keuls; Fig. 3a and b). Mirroring the data obtained *in vitro*, the LPS-induced change was significantly greater in tissue prepared from SIGIRR$^{-/-}$ mice ($^{+}p < 0.05$; $^{+++}p < 0.001$ versus LPS-treated wildtype mice). LPS also increased mRNA expression of the chemokines MCP-1 and IP-10 (*$p < 0.05$; Newman-Keuls; Fig. 3c and d), and these changes were further enhanced in SIGIRR$^{-/-}$ mice compared with wildtype mice ($^{+}p < 0.05$; versus LPS-treated wildtype mice). Similarly, while LPS increased (albeit non-significantly) CD80 and CD86, assessed by Western immunoblot in hippocampal tissue prepared from wildtype mice, the effect of LPS in SIGIRR$^{-/-}$ mice was statistically significant (*$p < 0.05$; Newman-Keuls; Fig. 3e and f). The data also indicate that MHCII immunoreactivity was increased in hippocampal sections prepared from SIGIRR$^{-/-}$, compared with wildtype mice, and the effect of LPS seemed to be slightly greater in SIGIRR$^{-/-}$ mice compared with wildtype mice (Fig. 3g).

*3.3. LPS induces a more robust increase in proinflammatory cytokines in hippocampus of*

*SIGIRR$^{-/-}$ mice*

Previous findings from this laboratory have coupled increased expression of cell surface markers of microglial activation and chemokines with increased production of proinflammatory cytokines (Clarke et al., 2008; Downer et al., 2010; Loane et al., 2007). Therefore we predicted that the enhanced response to LPS in tissue prepared from SIGIRR$^{-/-}$ mice would extend to production of proinflammatory cytokines. LPS markedly increased IL-6 and TNFα mRNA expression in hippocampus of wildtype mice (*$p < 0.05$; **$p < 0.01$; Newman-Keuls; Fig. 4a and b) and the effect was exacerbated in tissue prepared from SIGIRR$^{-/-}$ mice ($^{+}p < 0.05$; $^{++}p < 0.01$ versus LPS-treated wildtype mice). In parallel, and mirroring the data obtained *in vitro*, the significant LPS-induced increase in IL-6 concentration (*$p < 0.05$; Newman-Keuls; Fig. 4c) was more robust in hippocampal tissue prepared from SIGIRR$^{-/-}$ mice ($^{+++}p < 0.001$, versus LPS-treated wildtype mice). However LPS did not significantly increase TNFα concentration in hippocampal tissue prepared from either wildtype or SIGIRR$^{-/-}$ mice (Fig. 4d). These findings demonstrate that SIGIRR can function *in vivo* as a negative regulator of microglial activation and chemokine and cytokine production, in response to LPS. Furthermore, they provide the first evidence that the effects of SIGIRR are extended to the CNS.

*3.4. LPS-induced inhibition of exploratory behaviour is more profound in SIGIRR$^{-/-}$ mice*

It is well established that inflammatory changes in the brain lead to a constellation of non-specific symptoms collectively referred to as 'sickness behaviour', which is typified by a decrease in exploratory behaviour (Dantzer, 2004). An important objective of this study was to determine if exacerbated inflammation in the hippocampus of LPS-treated SIGIRR$^{-/-}$ mice was accompanied by enhanced sickness behaviour. To assess this, LPS-treated

wildtype and SIGIRR$^{-/-}$ mice were assessed in the open field test. Intraperitoneal injection of LPS, 30 minutes prior to testing, decreased the total distance travelled by 39% (**$p < 0.01$) and 60% (***$p < 0.001$; Newman-Keuls; Fig. 5a) in wildtype and SIGIRR$^{-/-}$ mice respectively. Head dipping was also significantly decreased in wildtype mice (*$p < 0.05$; Newman-Keuls; Fig. 5b) and SIGIRR$^{-/-}$ mice (**$p < 0.01$) and the LPS-induced effect in SIGIRR$^{-/-}$ mice was markedly greater than in wildtype animals ($^{+++}p < 0.001$). Importantly, control-treated SIGIRR$^{-/-}$ mice exhibited a reduction in exploratory behaviour, in terms of head dipping, than control-treated wildtype mice ($^{\S\S}p < 0.01$). LPS did not affect rearing in wildtype mice but significantly reduced it in SIGIRR$^{-/-}$ mice (***$p < 0.001$; Newman-Keuls; Fig. 5c).

Anxiety behaviour is reflected in the frequency of entries mice make into the centre portion of the apparatus and the mean time spent in the centre. LPS significantly decreased the frequency of entries in wildtype mice (**$p < 0.01$; Newman-Keuls; Fig. 5d) and SIGIRR$^{-/-}$ mice (***$p < 0.001$), and the LPS-induced effect in SIGIRR$^{-/-}$ mice was markedly greater than in wildtype animals ($^{+++}p < 0.001$). This measure was significantly decreased in control-treated SIGIRR$^{-/-}$ mice compared with control-treated wildtype mice ($^{\S\S}p < 0.01$). The mean percentage time (± SEM) spent in the centre of the apparatus was significantly decreased in SIGIRR$^{-/-}$, compared with wildtype mice ($p < 0.01$; Newman-Keuls) although LPS exerted no significant effect in either group of mice (data not shown). These findings, which reveal that SIGIRR$^{-/-}$ mice treated with LPS exhibited reduced exploratory behaviour in the open field test compared with wildtype mice, provide further evidence of a greater susceptibility of SIGIRR$^{-/-}$ mice to an LPS challenge.

*3.5. Age-related neuroinflammatory changes are exacerbated in hippocampus of SIGIRR$^{-/-}$*

*mice*

Age-related increases in the expression of proinflammatory cytokines have been reported by a number of groups including ours (Godbout and Johnson, 2004; Lynch and Lynch, 2002; Maher et al., 2005). Here, we argued that if SIGIRR exerts a tonic anti-inflammatory influence, then age-related increases in proinflammatory cytokine production will be exacerbated. Hippocampal tissue was prepared from young and aged wildtype and SIGIRR$^{-/-}$ mice and assessed for evidence of change in IL-6 and TNFα at mRNA and protein levels. While there was no significant age-related change in IL-6 or TNFα mRNA in hippocampus of wildtype mice, significant age-related increases were observed in tissue prepared from SIGIRR$^{-/-}$ mice (*$p < 0.05$; ***$p < 0.001$; Newman-Keuls; Fig. 6a, c). IL-6 concentration, assessed by ELISA, was significantly greater in tissue prepared from aged SIGIRR$^{-/-}$ mice, compared with aged wildtype mice ($^{+}p < 0.05$; Newman-Keuls; Fig. 6b). Although LPS increased mean hippocampal concentration of IL-6 from a basal value of 28.60 pg/mg ± 1.31 (SEM) to 169.5 pg/mg ± 59.24, in tissue prepared from wildtype mice, this did not reach statistical significance. TNFα concentration was significantly greater in hippocampal tissue prepared from aged, compared with young wildtype mice (***$p < 0.001$; Newman-Keuls; Fig. 6d) and in aged, compared with young SIGIRR$^{-/-}$ mice (***$p < 0.001$), although no additional effect of age was observed in tissue prepared from SIGIRR$^{-/-}$ animals.

*3.6. Expression of CD14 and TLR4 are increased in hippocampus of SIGIRR$^{-/-}$ mice*

Given that the responsiveness of SIGIRR$^{-/-}$ mice to LPS was upregulated, we argued that expression of TLR4 and/or CD14, which are necessary to trigger LPS signalling (Kennedy et al., 2004), would be increased in tissue prepared from SIGIRR$^{-/-}$ mice. In line with our

prediction, we determined that hippocampal mRNA expression of both was increased in tissue prepared from SIGIRR$^{-/-}$, compared with wildtype mice (*$p < 0.05$; two-tailed Student's t-test for independent means; Fig. 7a and b). We analysed expression of phosphorylated IκKα/β , as a measure of kinase activation, and phosphorylated IκBα as a measure of NFκB activation, by Western immunoblotting. Comparisons using Newman-Keuls tests did not detect statistically significant alterations between groups, however 2-way ANOVA revealed a marked increase in phosphorylated IκBα and IκKα/β in tissue prepared from SIGIRR$^{-/-}$, compared with wildtype mice (*$p < 0.05$; 2-way ANOVA; Fig. 7c and d). While LPS treatment did not produce an additional effect on either protein in wildtype or SIGIRR$^{-/-}$ mice (Fig. 7 c and d), a significant increase in both pIκBα and pIκKα/β was identified in aged, compared with young wildtype mice and aged, compared with young SIGIRR$^{-/-}$ mice (***$p < 0.001$; *$p < 0.05$; Newman-Keuls; Fig. 7e and f). Comparison by 2-way ANOVA also indicated this age-related effect. Interestingly, while the age-related effect was not exaggerated in SIGIRR$^{-/-}$ compared with wildtype mice, pIκBα was significantly lower in aged SIGIRR$^{-/-}$ relative to aged wildtype mice ($^{+}p < 0.05$; Newman-Keuls; Fig. 7e) although no interaction was determined by 2-way ANOVA. These data provide a mechanism by which the enhanced neuroinflammatory responses in SIGIRR$^{-/-}$ mice might be explained.

## 4. Discussion

We set out to establish whether the absence of SIGIRR endowed an inflammatory phenotype in the brain and to assess the functional consequences of its absence. The data

demonstrate that the inflammatory changes associated with age were enhanced in hippocampal tissue prepared from SIGIRR$^{-/-}$, compared with wildtype mice. Similarly the absence of SIGIRR was associated with accentuated LPS-induced increase in markers of microglial activation and inflammatory cytokine production. The data suggest that SIGIRR is an endogenous negative regulator of TLR4-induced signalling in brain, as it is in the periphery.

An anti-inflammatory role has been ascribed to SIGIRR in peripheral cells based largely on findings which indicate that in its absence, there is an exacerbated response to inflammatory mediators, and that overexpression of SIGIRR is associated with decreased LPS- and IL-1-induced signalling (Wald et al., 2003). The present data reveal that LPS induced release of IL-6 and TNFα from cultured glia obtained from wildtype mice, but that the effect of LPS was markedly enhanced in cells prepared from SIGIRR$^{-/-}$ mice. These data are consistent with the hypothesis that SIGIRR is a negative regulator of TLR signalling (Qin et al., 2005b; Wald et al., 2003) and specifically support the findings that the effect of stimuli, including LPS and IL-1, are exacerbated in SIGIRR-deficient kidney cells (Qin et al., 2005b) and in splenocytes (Wald et al., 2003), bone-derived macrophages and dendritic cells prepared from SIGIRR$^{-/-}$ mice (Garlanda et al., 2007; Garlanda et al., 2004). Consistent with this, overexpression of SIGIRR in RAW264.7 cells was associated with a decrease in expression of several proinflammatory cytokines (Huang et al., 2006). Analysis of changes in purified microglia and astrocytes revealed that the enhanced LPS-induced response in mixed glia prepared from SIGIRR$^{-/-}$ mice is attributable to a change in microglia rather than astrocytes.

In addition to its ability to stimulate cytokine release, LPS upregulates expression of cell surface proteins, considered to be markers of microglial activation (Loane et al., 2007; Qin et al., 2005a). In this study, LPS is shown to increase expression of CD40, as well as another cell surface marker of microglial activation, ICAM-1, but the effect of LPS was several-fold greater in cells prepared from SIGIRR$^{-/-}$ mice. Therefore in the brain, SIGIRR appears to provide tonic inhibition of TLR4-driven activity in glial cells, mirroring the data obtained in cells prepared from peripheral tissues (Lech et al., 2007; Wald et al., 2003). Consequently, activation of SIGIRR, by modulating the activation state of microglia, may be an important factor in preserving the balance between pro- and anti-inflammatory cytokine production.

Having established that LPS stimulated a greater response in cultured glia prepared from SIGIRR$^{-/-}$ mice, we then compared the effect of intraperitoneally-administered LPS in wildtype and SIGIRR$^{-/-}$ mice. We report that SIGIRR$^{-/-}$ mice have increased expression of the microglial markers CD40 and ICAM in response to LPS compared to wildtype mice; the co-stimulatory molecules CD80 and CD86 were also upregulated SIGIRR$^{-/-}$ mice in response to LPS and this effect was not observed in wildtype mice. As previously reported, LPS increased IL-6 and TNFα at mRNA and protein levels in hippocampus (Chen et al., 2008; Hellstrom et al., 2005; Loane et al., 2007; Sparkman et al., 2006); significantly, and in parallel with the changes *in vitro*, the effect of LPS was markedly greater in hippocampus of SIGIRR$^{-/-}$ mice. One factor which may contribute to these neuroinflammatory changes is infiltration of peripheral cells, as was shown in the ischaemic kidney (Lech et al., 2009). However, in the current study we were unable to detect evidence of leukocyte infiltration into the brain. Interestingly a lethal LPS challenge

was associated with increased mortality in SIGIRR$^{-/-}$, compared with wildtype mice (Wald et al., 2003). Other studies are largely consistent with these findings; for instance, deleting *Sigirr* in B6$^{lpr/lpr}$ mice (which develop very mild lupus erythematosus) resulted in development of severe disease characterized by evidence of significant inflammation (Lech et al., 2007) and production of proinflammatory mediators (Lech et al., 2008). Similarly post-ischaemic renal failure was worse in SIGIRR-deficient mice and this was accompanied by significant leukocyte transmigration in the microvasculature (Lech et al., 2009). Furthermore a similar, poorer outcome, linked with increased inflammation, was observed following fully mismatched kidney allotransplantation in SIGIRR-deficient mice (Noris et al., 2009). Experimentally-induced colitis, characterized by increased weight loss, intestinal bleeding, ulceration and/or mortality was also more profound in SIGIRR$^{-/-}$ mice (Garlanda et al., 2004; Xiao et al., 2007) as was colitis-associated cancer (Xiao et al., 2007). Histological analysis revealed dysregulation in turnover of gut epithelial cells in SIGIRR$^{-/-}$ mice, with increased proliferation and survival leading to elongated crypts over time; changes which were dependent on the presence of commensal bacteria (Xiao et al., 2007). These changes, and the increased susceptibility of SIGIRR$^{-/-}$ mice to colitis, were reversed when SIGIRR was reintroduced (Xiao et al., 2007). Another example of the modulating effect of SIGIRR on inflammation is the demonstration that treatment of mice with anti-SIGIRR antibody increased *P. aeruginosa*-induced inflammation in the cornea assessed by swelling, infiltration of inflammatory cells and production of proinflammatory cytokines (Huang et al., 2006).

LPS binds to the LPS binding protein and signals by means of interacting with CD14 (Kielian and Blecha, 1995), and following interaction with MD-2, provides the cascade by

which an inflammatory response is induced (Miggin et al., 2007). We considered that one possible mechanism which might explain the increased sensitivity of SIGIRR$^{-/-}$ mice to LPS may be increased expression of CD14 or TLR4 or both and the evidence presented here suggests that such an increase exists.

Although neither IL-6 mRNA nor TNFα mRNA were increased in hippocampal tissue prepared from aged, compared with young wildtype mice, hippocampal concentrations of both cytokines were increased in tissue prepared from aged wildtype mice compared with young mice; consistent with previous reports of age-related increases in these inflammatory markers (Godbout and Johnson, 2004; Lynch and Lynch, 2002; Maher et al., 2005). In addition to the increased responsiveness of SIGIRR$^{-/-}$ mice to an LPS challenge, there was a marked increase in the inflammatory cytokine IL-6 in hippocampal tissue prepared from aged SIGIRR$^{-/-}$ mice, compared with aged wildtype mice. Although it was shown by Qin and colleagues in 2005 that SIGIRR negatively regulates LPS signalling through its TIR domain by attenuating the recruitment of receptor-proximal signalling components to TLR4, the precise mechanism involved has only just been described. Using a computational approach it has been suggested that the key binding domain of SIGIRR is the BB loop; SIGIRR binds to TLR4 and 7 by occupying their self-interacting sites, it also appears to disrupt the formation of the MyD88 homodimer by replacing a MyD88 monomer (Gong et al.).

LPS has been shown to induce sickness behaviour (Konsman et al., 2002) and an important objective of this study was to determine if the accentuated LPS-induced changes in inflammatory markers in the hippocampus of SIGIRR$^{-/-}$ mice were associated with evidence of behavioural changes. We demonstrate that LPS treatment decreased

locomotion in the open field; the total distance covered and the velocity were both decreased which generally concurs with results of previous studies (Konsman et al., 2002; Swiergiel and Dunn, 2007). These adverse effects of LPS treatment were similar in wildtype and SIGIRR$^{-/-}$ mice. LPS also markedly decreased exploratory behaviour in wildtype and SIGIRR$^{-/-}$ mice; both head dipping and rearing behaviour were decreased and LPS-treated mice entered the central zone fewer times than controls. However in each of these measures, the effect of LPS was more profound in SIGIRR$^{-/-}$ mice, and indeed exploratory behaviour was decreased even in control-treated SIGIRR$^{-/-}$ mice. These findings are consistent with previous evidence of increased effects of LPS in SIGIRR$^{-/-}$ mice (Wald et al., 2003). Control-treated SIGIRR$^{-/-}$ mice spent less time exploring the central zone compared with control-treated wildtype mice suggesting that a deficiency in the SIGIRR gene may increase anxiety. While the effects of LPS treatment on microglial markers and cytokines were measured 3 hours post-injection, wildtype and SIGIRR$^{-/-}$ mice were assessed in the open field test 30 minutes following LPS injection. This experimental regime was chosen as preliminary experiments demonstrated that 3 hours following LPS injections SIGIRR$^{-/-}$ mice were motionless and thus rendered unable to participate in a stringent motor test such as the open-field test.

The findings presented here add to the considerable body of evidence indicating that SIGIRR modulates inflammatory responses in a variety of tissues (Garlanda et al., 2009) . The data provide the first indication that SIGIRR acts as an endogenous negative regulator of inflammation in the brain, and that its loss exaggerates the age-related upregulation in cytokine production in the hippocampus. SIGIRR also plays a significant role in modulating the response of the brain to inflammatory stimuli, and the data

presented suggest that the increased responsiveness to LPS may derive from upregulated expression of CD14 and TLR4, and increased activation of NFκB in response to SIGIRR deficiency. Further study using animal models of disease should highlight whether SIGIRR may indeed be a potentially valuable therapeutic target.

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## Figure legends

**Fig. 1.** The effect of LPS on markers of microglial activation is enhanced in SIGIRR$^{-/-}$ mice. LPS (100ng/ml; 24 hours) significantly increased CD40 mRNA (a) and ICAM

mRNA (b) expression in glial cells prepared from wildtype and SIGIRR$^{-/-}$ mice (*$p < 0.05$; ***$p < 0.001$; Newman-Keuls; n=3-6). Treatment with LPS induced a significantly greater effect in cells prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice ($^{+++}p < 0.001$; Newman-Keuls; n=4-6). A 2-way ANOVA revealed a significant interaction of LPS treatment and SIGIRR$^{-/-}$ on CD40 mRNA [a; $F(1,15) = 8.57$; $p < 0.05$] and ICAM mRNA [b; $F(1,15) = 15.61$, $p < 0.005$].

**Fig. 2.** LPS-induced release of pro-inflammatory cytokines is enhanced in SIGIRR$^{-/-}$ mice. LPS (100ng/ml; 24 hours) increased the concentrations of IL-6 (a,c,e) and TNFα (b,d,f) measured by ELISA in mixed glial cells (a,b), purified astrocytes (c,d) and purified microglia (e,f) prepared from wildtype and SIGIRR$^{-/-}$ mice (***$p < 0.001$; Newman-Keuls; n=4-10). Treatment with LPS significantly enhanced these effects in mixed glia and microglia, but not astrcoytes, prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice ($^{++}p < 0.01$, $^{+++}p < 0.001$; Newman-Keuls; n=4-6). A 2-way ANOVA identified a significant interaction of LPS and SIGIRR$^{-/-}$ on both IL-6 [a; $F(1,31) = 6.39$; $p < 0.05$] and TNFα production [b; $F(1,34) = 7.99$; $p < 0.01$] in mixed glial cells. While a significant LPS effect was also observed on IL-6 in purified astrocytes [c; $F(1,17) = 683.0$; $p < 0.0001$], an interaction of LPS and SIGIRR$^{-/-}$ was identified in purified microglia [e; $F(1,15) = 428.9$; $p < 0.0001$]. Similarly 2-way ANOVA revealed significant effect of LPS on TNFα in purified astrocytes [d; $F(1,18) = 478.8$; $p < 0.0001$] , while a significant interaction of LPS and SIGIRR$^{-/-}$ was observed in isolated microglia [f; $F(1,15) = 1049.0$; $p < 0.0001$].

**Fig. 3.** LPS-induced expression of microglial activation markers is enhanced in SIGIRR$^{-/-}$ mice. Intraperitoneal injection of LPS (10μg/kg) significantly increased CD40 mRNA (a), ICAM mRNA (b), MCP-1 mRNA (c) and IP-10 mRNA (d) expression in hippocampal tissue prepared from wildtype and SIGIRR$^{-/-}$ mice (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; Newman-Keuls; n=6), and this LPS-induced effect was significantly enhanced in hippocampal tissue prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice ($^{+}p < 0.05$; $^{+++}p < 0.001$; Newman-Keuls; n=6). A 2-way ANOVA revealed a significant effect of LPS on CD40 mRNA [a; $F(1,16) = 19.79$; $p < 0.0005$], MCP-1 mRNA [c; $F(1,18) = 38.14$; $p < 0.0001$] and IP-10 mRNA [d; $F(1,19) = 47.94$; $p < 0.0001$] expression, while there was a significant LPS and SIGIRR$^{-/-}$ interaction on the expression of ICAM mRNA [b; $F(1,17) = 75.75$; $p < 0.0001$]. (e,f) CD80 and CD86 were both significantly increased by LPS treatment in hippocampal tissue prepared from SIGIRR$^{-/-}$ mice (*$p < 0.05$; Newman-Keuls), but not wildtype mice. A 2-way ANOVA also demonstrated an LPS-mediated effect on CD80 [e; $F(1,20) = 9.55$; $p < 0.01$] and CD86 [f; $F(1,19) = 10.24$, $p < 0.005$]. (g) MHCII immunoreactivity was greater in hippocampal sections prepared from SIGIRR$^{-/-}$, compared with wildtype mice and the effect of LPS appeared greater in sections prepared from SIGIRR$^{-/-}$ mice.

**Fig. 4.** The effect of LPS on proinflammatory cytokines is increased in hippocampus of SIGIRR$^{-/-}$ mice. Intraperitoneal injection of LPS (10μg/kg) significantly increased IL-6 mRNA (a) and TNFα mRNA (c) as assessed by PCR in hippocampal tissue prepared from wildtype and SIGIRR$^{-/-}$ mice (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; Newman-Keuls; n=5-12). Similarly, the concentration of IL-6 (b) as assessed by ELISA, was significantly

increased in hippocampal tissue from wildtype and SIGIRR$^{-/-}$ mice (*p < 0.05; **p < 0.01; Newman-Keuls; n=5-6) following LPS treatment. In addition, the LPS-induced effects were significantly enhanced in hippocampus of SIGIRR$^{-/-}$ mice relative to wildtype animals ($^{+}$p < 0.05; $^{++}$p < 0.01; $^{+++}$p < 0.001; Newman-Keuls; n=5-12). No significant changes in TNFα concentration were observed (d). 2-way ANOVA determined a significant effect of LPS on IL-6 mRNA [a; $F(1,39) = 14.76$; $p < 0.0005$] and TNFα mRNA [c; $F(1,19) = 12.95$; $p < 0.005$] along with a significant SIGIRR$^{-/-}$ mediated effect on IL-6 mRNA [a; $F(1,39) = 4.20$; $p < 0.05$]. A significant LPS and SIGIRR$^{-/-}$ interaction was also observed on IL-6 protein concentration [b; $F(1,18) = 5.08$; $p < 0.05$], while there were no significant effects on TNFα concentration.

**Fig. 5.** Sickness behaviour is exacerbated in SIGIRR$^{-/-}$ mice. LPS significantly reduced total distance travelled (a), head dipping (b) and the number of entries into the central zone of a hole-board (d), but not rearing (c) in wildtype mice (*p < 0.05; **p < 0.01; Newman-Keuls; n=6). Both head dipping (b) and the number of entries into the central zone of the hole-board (d) were significantly reduced in control-treated SIGIRR$^{-/-}$ mice compared with control-treated wildtype mice ($^{\$\$}$p < 0.01; Newman-Keuls; n=6). LPS exerted a significantly greater effect on head dipping and the number of entries into the central zone SIGIRR$^{-/-}$ mice compared with wildtype mice ($^{+++}$p < 0.001; Newman-Keuls; n=6). While LPS exerted no effect on rearing in wildtype mice (c), it was significantly reduced in SIGIRR$^{-/-}$ mice (***p < 0.001; Newman-Keuls; n=6). 2-way ANOVA identified an LPS-induced effect on all parameters tested; total distance travelled [a; $F(1,19) = 32.26$; $p < 0.001$], head dipping [b; $F(1,19) = 22.40$; $p < 0.0005$], rearing [c;

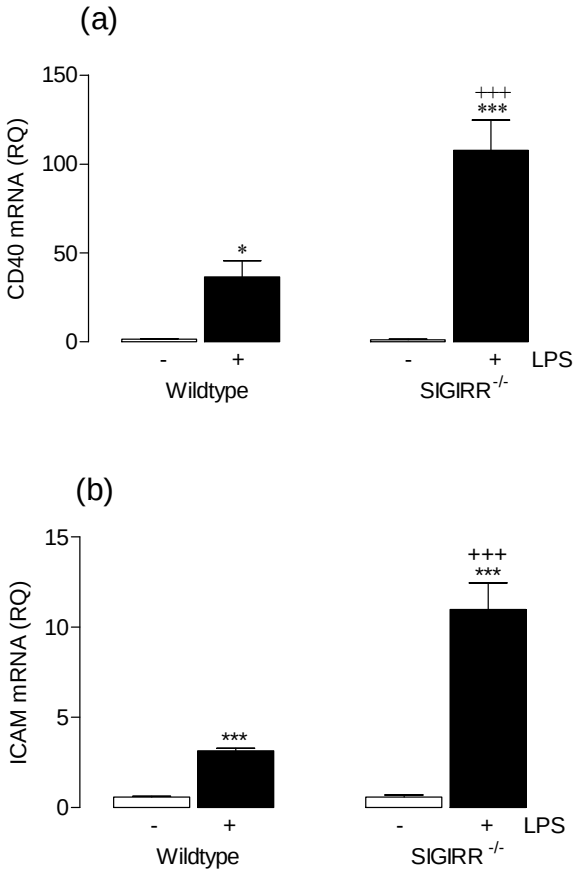
$F(1,19) = 10.85$; $p < 0.005$] and number of entries into the central zone [d; $F(1,18) = 45.53$; $p < 0.0001$]. A SIGIRR$^{-/-}$ mediated effect was also determined for head dipping [b; $F(1,19) = 39.24$; $p < 0.0001$] and the number of entries into the central zone [d; $F(1,18) = 35.36$; $p < 0.0001$].

**Fig. 6.** Pro-inflammatory cytokines are increased in hippocampus of aged SIGIRR$^{-/-}$ mice. IL-6 mRNA (a) and TNFα mRNA (c) expression, as assessed by PCR, were significantly increased in hippocampal tissue prepared from aged SIGIRR$^{-/-}$ mice compared with young SIGIRR$^{-/-}$ mice (*$p < 0.05$; ***$p < 0.001$; Newman-Keuls; n=4-6) and compared with aged wildtype mice ($^{+}p < 0.05$; $^{++}p < 0.01$; Newman-Keuls; n=4-6). 2-way ANOVA confirmed a significant interaction of age and SIGIRR$^{-/-}$ on both IL-6 mRNA [a; $F(1,36) = 24.51$; $p < 0.0001$] and TNFα mRNA [c; $F(1,14) = 5.68$; $p < 0.05$]. Hippocampal concentration of TNFα (d) was significantly increased in hippocampal tissue prepared from aged wildtype and SIGIRR$^{-/-}$ mice compared with young mice. IL-6 concentration (b) was significantly increased in aged SIGIRR$^{-/-}$ mice (**$p < 0.01$; ***$p < 0.001$; Newman-Keuls; n=4-6), and the age-associated effect in IL-6 was enhanced in hippocampal tissue prepared from SIGIRR$^{-/-}$ mice compared with aged wildtype mice ($^{+}p < 0.05$; Newman-Keuls; n=4-6). 2-way ANOVA revealed a significant effect of age on IL-6 [b; $F(1,17) = 9.28$; $p < 0.01$] and TNFα [d; $F(1,17) = 49.92$; $p < 0.0001$] protein concentrations.

**Fig. 7.** Expression of CD14 and TLR4, and phosphorylation of IκBα and IκKα/β, are increased in hippocampus of SIGIRR$^{-/-}$ mice. CD14 mRNA (a) and TLR4 mRNA (b)

expression were significantly increased in hippocampal tissue prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice (*$p < 0.05$; two-tailed student t-test; n=5-6). While *post hoc* analysis using Newman-Keuls tests did not identify differences in phosphorylated IκBα (c) and IκKα/β (d) in wildtype and SIGIRR$^{-/-}$ mice in response to LPS, 2-way ANOVA revealed that expression of pIκBα [c; $F(1,30) = 6.589$; *$p < 0.05$; n=6-11] and pIκKα/β [d; $F(1,30) = 4.242$; *$p < 0.05$; n=6-11] was significantly increased in tissue prepared from SIGIRR$^{-/-}$ mice compared with wildtype mice. Additionally, expression of phosphorylated IκBα and IκKα/β was significantly increased in hippocampus of aged, compared with young wildtype mice (e,f; $^{*}p < 0.05$; ** $p < 0.01$; Newman-Keuls; n=4-6) and aged, compared with young SIGIRR$^{-/-}$ mice (e,f; $^{*}p < 0.05$; Newman-Keuls; n=4-9). A 2-way ANOVA also identified a significant age-related increase in pIκBα [e; $F(1,19) = 29.71$; $p < 0.0001$] and pIκKα/β [f; $F(1,19) = 25.50$; $p < 0.0001$] in hippocampal tissue from wildtype and SIGIRR$^{-/-}$ mice. While phosphorylated IκBα was significantly lower in aged SIGIRR$^{-/-}$, compared with aged wildtype mice (e; $^{+}p < 0.05$; Newman-Keuls; n=4), no significant interaction of age and SIGIRR$^{-/-}$ was identified using 2-way ANOVA.

# Figure 1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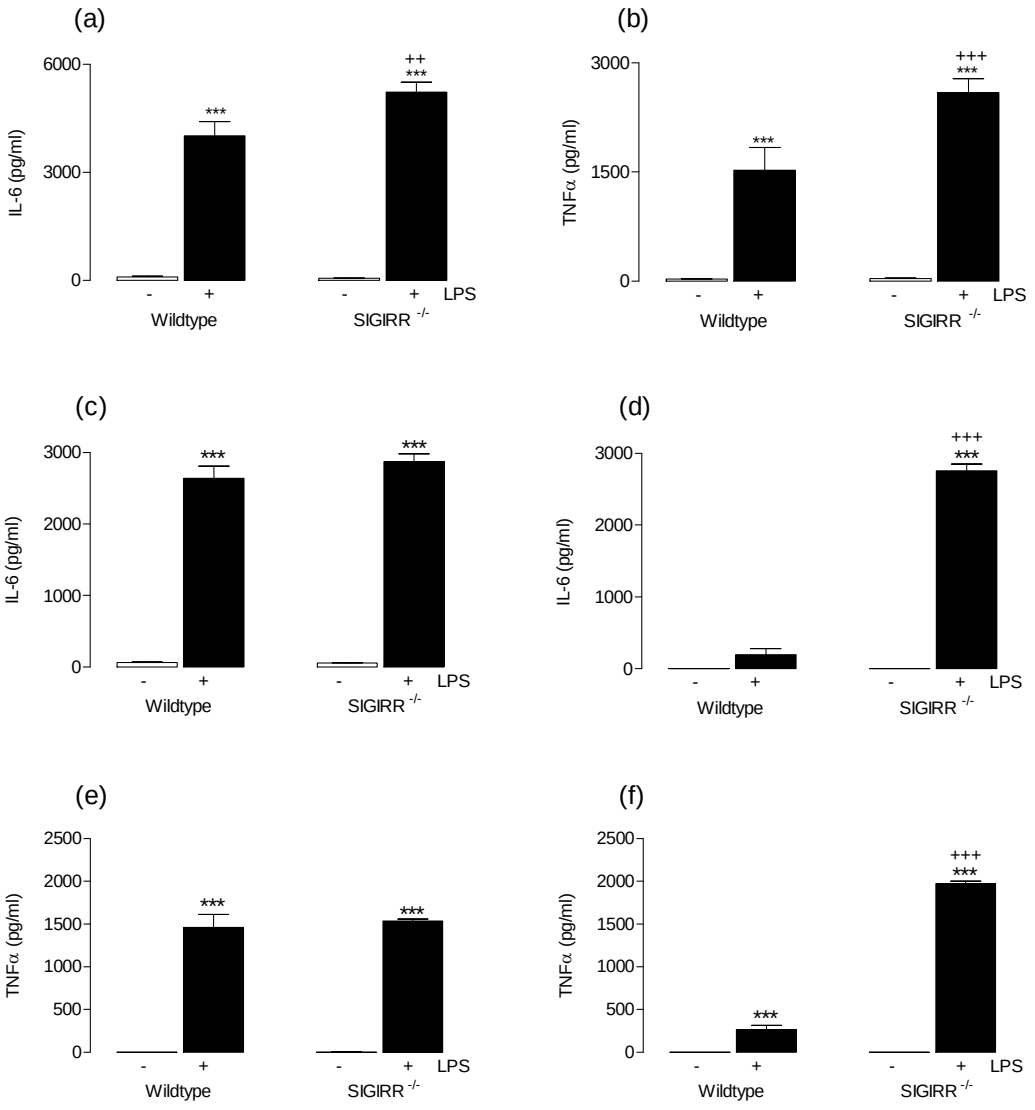
# Figure 2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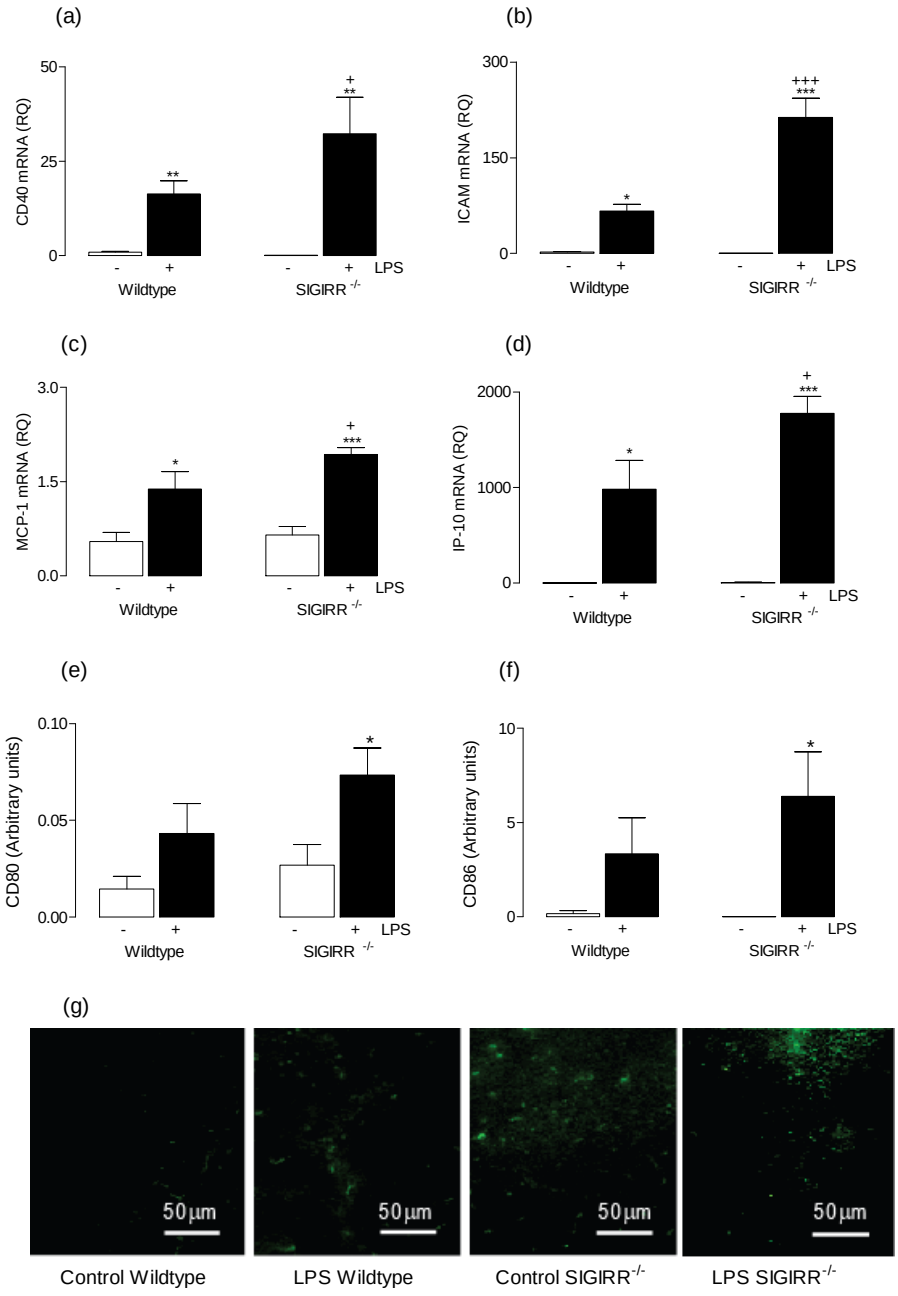
# Figure 3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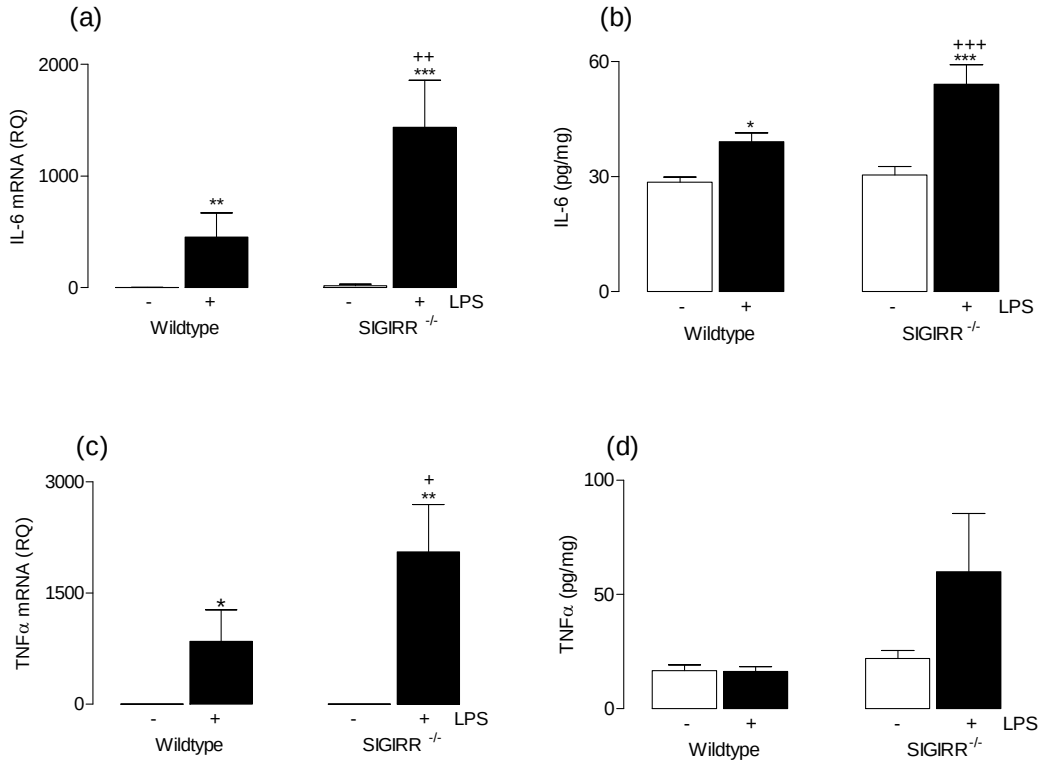
# Figure 4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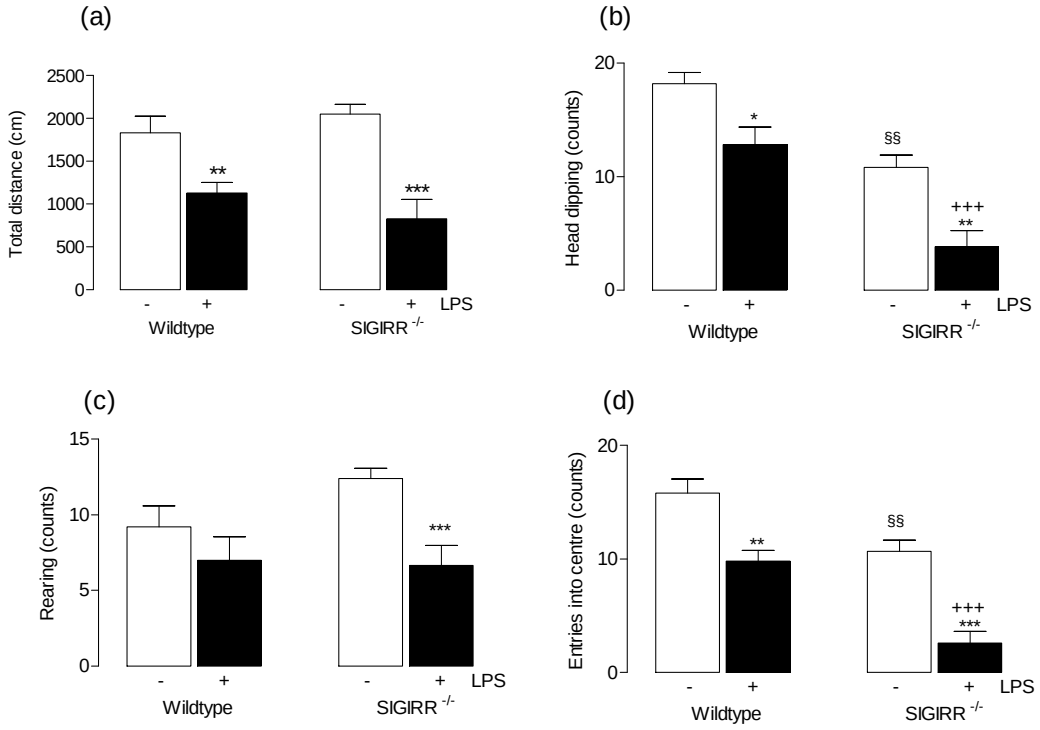
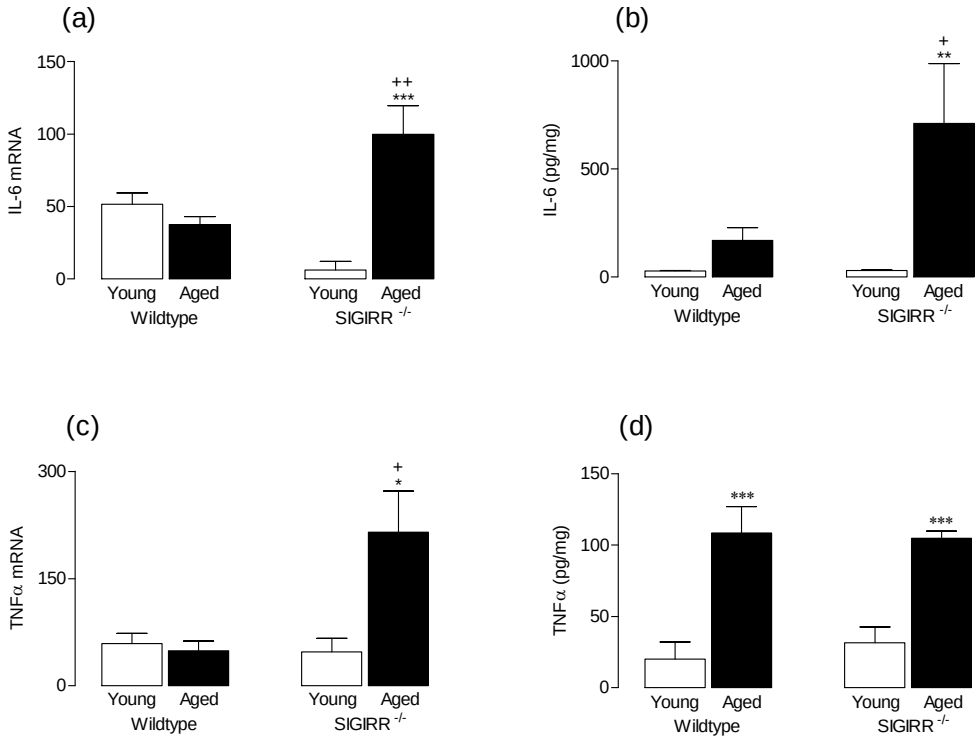
- + LPS
Wildtype
SIGIRR -/-
(d)
TNFα (pg/mg)
100
50
0
- + LPS
Wildtype
SIGIRR -/-

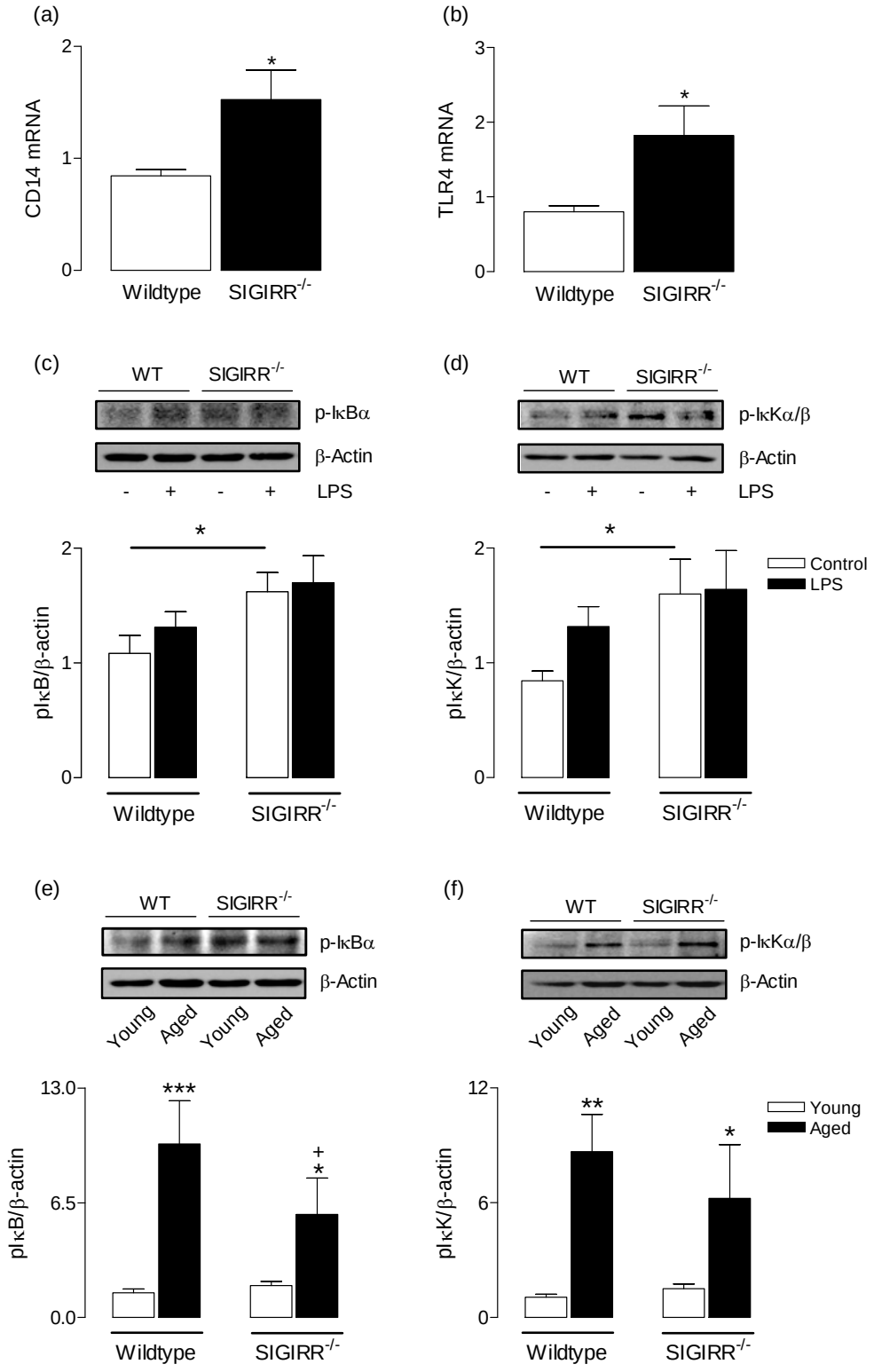
# Figure 5


(a)
Total distance (cm)
2500
2000
1500
1000
500
0
**
***
-
+
LPS
Wildtype
SIGIRR -/-
(b)
Head dipping (counts)
20
10
0
*
§§
+++
**
(c)
Rearing (counts)
15
10
5
0
***
(d)
Entries into centre (counts)
20
10
0
**
§§
+++
***

# Figure 6


(a)
IL-6 mRNA
150
100
50
0
++
***
Young
Aged
Wildtype
Young
Aged
SIGIRR -/-
(b)
IL-6 (pg/mg)
1000
500
0
+
**
Young
Aged
Wildtype
Young
Aged
SIGIRR -/-
(c)
TNFα mRNA
300
150
0
+
*
Young
Aged
Wildtype
Young
Aged
SIGIRR -/-
(d)
TNFα (pg/mg)
150
100
50
0
***
***
Young
Aged
Wildtype
Young
Aged
SIGIRR -/-

# Figure 7


(a)
CD14 mRNA
Wildtype
SIGIRR-/-
(b)
TLR4 mRNA
Wildtype
SIGIRR-/-
(c)
WT
SIGIRR-/-
p-IκBα
β-Actin
- + - +
LPS
pIκB/β-actin
Wildtype
SIGIRR-/-
(d)
WT
SIGIRR-/-
p-IκKα/β
β-Actin
- + - +
LPS
pIκK/β-actin
Control
LPS
Wildtype
SIGIRR-/-
(e)
WT
SIGIRR-/-
p-IκBα
β-Actin
Young
Aged
Young
Aged
pIκB/β-actin
Wildtype
SIGIRR-/-
(f)
WT
SIGIRR-/-
p-IκKα/β
β-Actin
Young
Aged
Young
Aged
pIκK/β-actin
Young
Aged
Wildtype
SIGIRR-/-