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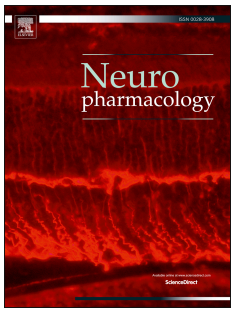

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EBI2 regulates pro-inflammatory signalling and cytokine release in astrocytes.

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Short title: *EBI2/oxysterol pro-inflammatory signalling in the CNS*

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Abstract

The endogenous oxysterol 7α, 25-dihydroxycholesterol (7α25HC) ligand activates the G protein-coupled receptor EBI2 to regulate T cell-dependant antibody response and B cell migration. We have demonstrated that EBI2 is expressed in human and mouse astrocytes, that 7α25HC induces intracellular signalling and astrocyte migration, and that EBI2 plays a role in the crosstalk between astrocytes and macrophages. Recently, we demonstrate that EBI2 regulates myelin development and inhibits LPC-induced demyelination. Here, we show that 7α25HC inhibits LPS- and IL17/TNF-induced pro-inflammatory cytokine release in astrocytes. We observe the following: 1. human astrocytes treated with IL17/TNF increases the nuclear translocation of NFκB, which is attenuated by pre-treatment with 7α25HC; 2. IL17/TNF increases cell impedance in human astrocytes, which is also attenuated by pre-treatment with 7α25HC; 3. the EBI2 antagonist NIBR189 inhibits these effects of 7α25HC, supporting the role of EBI2; 4. *in vivo* data corroborate these *in vitro* findings, showing that EBI2 knock-out (KO) animals display enhanced pro-inflammatory cytokine in response to LPS challenge, in the brain. These results demonstrate a role for oxysterol/EBI2 signalling in attenuating the response of astrocytes to pro-inflammatory signals as well as limiting the levels of pro-inflammatory cytokines in the brain.

Key words: Epstein-Barr virus induced gene 2 (EBI2); 7α,25-OHC; oxysterols; astrocytes; pro-inflammatory cytokines; intracellular signalling; LPS challenge; brain cytokine levels.

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INTRODUCTION

The G protein-coupled receptor EBI2 (Epstein Barr virus-induced gene 2, GPR183) is activated by the oxysterol 7α25HC (7α, 25-dihydroxycholesterol). This ligand is synthesised from cholesterol by cholesterol 25-hydroxylase (CH25H) and cytochrome P450 oxysterol 7-alpha-hydroxylase (CYP7B1), and is degraded by cholest-5-ene-3β,7α-diol 3β-dehydrogenase (HSD3B7) (Russell, 2000; Yi et al., 2012). EBI2 is expressed in peripheral blood mononuclear cells (Birkenbach et al., 1993; Hannedouche et al., 2011; Liu et al., 2011; Russell, 2000), plays a crucial role in B cell positioning in lymphoid tissue, enhances T follicular helper (Tfh) cells survival rate as well as T cell-dependant antibody response (Gatto et al., 2011; Li et al., 2016; Pereira et al., 2009). A functional role of the oxysterol/EBI2 pathway has also been shown in bone metastatic cancer where EBI2 guides osteoclast precursors to the bone (Nevius et al., 2015). Moreover, the EBI2 signalling pathway appears to be a sensor of immune challenge, where for example, lipopolysaccharide (LPS) exposure promoted EBI2 signalling in B cells, macrophages, and other immune cells by upregulating the expression of EBI2, CYP7B1 and CH25H, while decreasing HSD3B7 (Diczfalusy et al., 2009; Preuss et al., 2014; Rutkowska et al., 2016b). This LPS-mediated immune challenge also increases the levels of 25HC (the precursor to 7α25HC) in the humans and mice (Bauman et al., 2009; Diczfalusy et al., 2009), which can inhibit activity of viruses such as HIV, herpes and Ebola (Liu et al., 2013).

Oxysterols exert both, pro- and anti-inflammatory effects depending on the type of oxysterol and cell type in question (Aye et al., 2012; Fowler et al., 2003; Kim et al., 2006; Koarai et al., 2012). Oxysterols upregulate expression of adhesion molecules (ICAM-1, VCAM-1), growth factors (TGFβ1), cytokines (IL8) and chemokines (MCP1) involving ERK and NFκB signalling pathways (Lemaire et al., 1998; Leonarduzzi et al., 2005; Leonarduzzi et al., 2001; Otaegui-Arrazola et al., 2010). In the case of 25HC, this oxysterol enhances the release of pro-inflammatory cytokines from placental throphoblasts, airway epithelial cells, monocytic THP-1 cells and macrophages (Aye et al., 2012; Diczfalusy et al., 2009; Koarai et al., 2012; Lemaire-Ewing et al., 2009; Rosklint et al., 2002). On the other hand, oxidised low density lipoprotein and several oxysterols (7βHC, 24SHC, 25HC and 27HC) inhibit TNFα and IL1β levels in macrophages via NFκB and AP-1 transcription factors (Englund et al., 2001; Ohlsson et al., 1996). Moreover, the oxysterol 7β25HC, has immunosuppressive effects in lymphocytes (Moog et al., 1988) where it activates EBI2 to negatively regulate IFNγ response and cytokine levels (Chiang et al., 2013). Unsurprisingly, therefore, macrophages

deficient in CH25H enzyme produce greater amounts of IL1β and CH25H null mice exhibit exacerbated course of experimental autoimmune encephalomyelitis (EAE) (Reboldi et al., 2014). We note, there are contradictory findings also, where CH25H knock-out mice used in EAE display attenuated disease progression by limiting the influx of CD4+ T cells into the CNS (Chalmin et al., 2015). Interestingly, dysregulated levels of various oxysterols as well as EBI2 have been reported in a number of neurological diseases such multiple sclerosis (MS), Alzheimer's disease or cerebrotendinous xanthomatosis (Clottu et al., 2017; Crick et al., 2016; Leoni et al., 2004; Lutjohann et al., 2000; Panzenboeck et al., 2007; Wanke et al., 2017).

In addition to playing important roles in the immune system, EBI2 is also expressed in the cells of the central nervous system (CNS), namely astrocytes, and this receptor regulates astrocyte signalling, as well as astrocyte cell migration (Rutkowska et al., 2016a; Rutkowska et al., 2015). Moreover, it has been shown that LPS induces the release of oxysterols such as 25HC, 7α25HC and 7β25HC in astrocytes and that the oxysterol/EBI2 signalling pathway is involved in the crosstalk between the immune cells, namely macrophages, and the CNS cells, namely astrocytes (Rutkowska et al., 2016b). Most recently, we have demonstrated that EBI2 regulates myelin development and inhibits LPC-induced demyelination (Rutkowska et al., 2017). Therefore, in the immune system as well as the CNS, oxysterols and EBI2 signalling appears responsive to pro-inflammatory signals including, TLR4 stimulation or type I/II interferons, which may provide a mechanism against infection and neuroinflammation (Blanc et al., 2013; Diczfalusy et al., 2009; Rutkowska et al., 2016b). Here, to investigate further the glial cell functions of EBI2, we examined its role in regulating the response to and release of pro-inflammatory cytokines in human and rodent astrocytes, and *in vivo* using EBI2 knockout mice.

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MATERIALS AND METHODS

Cell Culture and Cytokine Analysis

All animal procedures were performed according to the national and institutional ethical guidelines. Human, mouse and rat astrocytes were cultured as we have described before (Elain et al., 2014; Healy et al., 2013; Mullershausen et al., 2007; Osinde et al., 2007). Prior to stimulation, astrocytes were starved in serum-free media for 4 hours (h). Astrocytes were then treated with IL17 (50 ng/ml, R&D Systems, 317-ILB)/TNFα (10 ng/ml, R&D Systems, 210-TA) or LPS (100 or 10 ng/ml, Sigma) with or without 7α25HC (prepared as 10 mM stock in 90% DMSO) or EBI2 antagonist NIBR189 (prepared as 10 mM stock in 90% DMSO). After 18-20 h cytokine analysis was performed using the conditioned media. Human Cytokine Array Panel A (ARY005, R&D Systems), Mouse Cytokine Array Kit Panel A (ARY006, R&D Systems) and Rat Cytokine Array Kit Panel A (ARY008, R&D Systems) were used as we have described previously (Sheridan and Dev, 2011). Cytokines were also measured with the R&D Systems ELISA (pg/ml) for human IL6 (DY206), mouse IL6 (DY406), mouse TNFα (DY410), rat IL-6 standard ABTS ELISA kit (900-K86, Peprotech), mouse TNF or were measured using homogeneous time resolved fluorescence (HTRF) (ng/ml) with human IL6 kit (Cisbio, 62IL6PEB), also as we have described before (Elain et al., 2014; Sheridan and Dev, 2011)

Animals

Studies described in this report were approved by the Swiss Cantonal Veterinary Authority of Basel City, Switzerland, under the license number 2644. The mixed genetic background (C57BL/6 x SV129) EBI2 double (-/-) knock-out (KO) mouse strain was obtained from Deltagen (Hannedouche et al., 2011) and backcrossed several generations to C57BL/6. The KO and wild-type (WT) littermates were housed in IVC racks (max. 4 mice/XJ Type cage) under specified pathogen-free conditions with *ad libitum* access to standard diet and water. Before euthanasia animals were perfused transcardially by phosphate-buffered saline (PBS) under isoflurane anaesthesia. The brains were then isolated and deep frozen.

Real Time Quantitative Polymerase Chain Reaction (RT-qPCR)

For mRNA expression analysis, after treatment, astrocytes were washed twice with PBS, scraped, resuspended in RA1 lysis buffer (Machery-Nagel, 740961) supplemented with 10% β-mercapthoethanol and frozen at -80^{o}C. RNA was isolated using the RNeasy Mini Kit (Qiagen Hilden, Germany). After reverse transcription of mRNA (10 min, 25°C; 120 min,

37°C; 5 sec, 85°C) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany), qRT-PCR was performed with the 7900HT Fast Real-Time PCR System (Applied Biosystems) according to the Standard Thermal Cycler Protocol (2 min, 50°C; 10 min, 95°C; 40 cycles 15 sec, 95°C and 1 min, 60°C). The following FAM dye-labeled TaqMan mouse probes were used: IL6 (Hs00985639-m1), HPRT1 (Hs02800695-m1) (Applied Biosystems). Each condition was run in quadruplicates, with each quadruplicate RNA sample run in duplicates and the experiments were repeated three times. The threshold was set manually for all samples. The analysis was performed with the SDS 2.3 software. The relative expression of IL6 to the reference gene (HPRT1) was determined.

Immunocytochemistry and NFκB Studies

Human astrocytes were washed twice with PBS, fixed with fixation/permeabilization kit (BD Cytofix/Cytoperm, 554714) for 20 min on ice, and then washed twice with PBS. The cells were incubates for 1 h at room temperature in TwB blocking buffer (PBS, 0.1% Tween-20, 1% BSA). For all antibody incubation and wash steps TwB* (PBS, 0.05% Tween-20, 0.5% BSA) was used. The cells were incubated overnight at 4^{o}C with anti-NFκB antibody (Santa-Cruz, sc-109), washed twice and incubated with secondary antibody Alexa goat anti-mouse 488 (Invitrogen, A11029) for 1 h. The cells were then washed twice, incubated with the nuclear stain Hoechst 34580 (Invitrogen, H21486) in PBS for 10 min, washed twice again with PBS and visualized with a Zeiss LSM 700 confocal microscope.

xCELLigence Impedance Assay

Impedance measurements of human astrocytes were conducted using E-plate 96 (ACEA Biosciences, USA) and recorded with the Real-Time Cell Analyzer (RTCA) Cardio Instrument (ACEA Biosciences, USA). The RTCA station was installed inside a standard culture incubator (37^{o}C with 5% CO_2) and the remaining equipment was located outside. The bottom of each well in an E-plate is covered with an array of electrodes that detect changes in cell impedance. The electronic impedance detects changes of cells attachment to the surface of the electrodes. Impedance value or parameter called cell index (CI) is given to represent the changes in electrical impedance detected during measurement and automatically calculated according to the following formula: CI=(Zi-Z0)/15 (where, Zi is the impedance at a single time point and Z0 is the baseline impedance measured at the start of the experiment). The CI values change in response to cell movement, morphological changes induced by compounds, cell proliferation, cell death, strength of adhesion or cell spread. After plating,

the cells were allowed to reach 80% confluence and then starved for 4 h followed by 30 min pre-treatment with EBI2 antagonist NIBR189 (1µM) (Gessier et al., 2014), followed by 30 min pre-treatment with 7α25HC (1 µM) and then combined treatment with IL17 (50 ng/ml) and TNFα (10 ng/ml). The impedance measurements were taken every 15 min. All conditions were run in quadruplicates.

LPS Challenge Model and Cytokine Analysis

Animals were treated intraperitoneally (i.p.), once daily (qd) with 0.5 mg/kg LPS from Salmonella abortus equi S-form (Enzo LifeSciences) or vehicle (PBS) for four consecutive days. Body weight was checked daily. At day 4 animals were sacrificed 3 hours after last LPS injection with PBS perfusion. Brains were removed and deep frozen. Brains were homogenized in RIPA buffer (Sigma) containing protease inhibitors (complete Mini, Roche) at 1:10 w/v in Precyellys24 tubes (CK28-R). Supernatant was used for analysis in the proinflammatory panel 1 (mouse) kit (V-plex, K15245D, MSD) at a dilution of 1:2 in reagent diluent 41 according to the manufactures protocol.

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RESULTS

7α25HC attenuates LPS- and IL17/TNFα-induced levels of pro-inflammatory cytokines in astrocytes

Given that oxysterols inhibit inflammatory responses in LPS stimulated microglia (Kim et al., 2006), we examined whether the oxysterol 7α25HC, via its receptor EBI2, has similar roles in astrocytes. Treatment with LPS (10 ng/ml or 100 ng/ml, 18 hours) induced a 10-fold increase in IL6 levels, with no significant difference between both LPS concentrations **(Fig 1a)**, while pre-treatment with 7α25HC partially inhibited LPS-induced IL6 release in a concentration dependent manner with 1 µM 7α25HC being the most potent (68.4%+/-13.1%), followed by 0.1 µM (85.8%+/-7.5%) and 0.01 µM (88.1%+/-13.1%) **(Fig 1b)**. Based on this data, for subsequent experiments we pre-treated mouse **(Fig 1c, d)** or human astrocytes **(Fig 1e, f)** with 7α25HC 1 µM for 30 minutes followed by LPS 100 ng/ml for 18 hours. Pre-treatment of mouse astrocytes with 7α25HC (1 µM) showed a modest but significant attenuation of LPS-induced levels of IL6 (90.1%+/-0.4%) **(Fig. 1c)** and also inhibited the levels of TNFα (75.3%+/-11.4%) **(Fig. 1d)**. Immunostaining of these astrocyte cultures showed no observable contamination with microglia as determined by Iba1 immunostaining **(Supplemental Fig. 1)**, suggesting that LPS directly induced astrocytes to increase levels of the cytokines. To further corroborate the direct role of EBI in astrocytes, we employed the use of pure human astrocyte cultures, as we have used previously (Elain et al., 2014; Healy et al., 2013; O'Sullivan and Dev, 2015; O'Sullivan et al., 2016; O'Sullivan and Dev, 2017; O'Sullivan et al., 2017; Rutkowska et al., 2016b; Rutkowska et al., 2015; Rutkowska et al., 2017). Previous studies show that human astrocytes do not respond to LPS (**Supplemental Fig 2**). due to low or no expression of TLR4 (Tarassishin et al., 2014), but release IL6 after treatment with IL17/TNFα (Elain et al., 2014; O'Sullivan et al., 2016; Tarassishin et al., 2014). The pre-treatment of human astrocytes with 7α25HC (1 µM, 30 minutes) showed a modest but significant decrease in IL17/TNFα (50/10 ng/ml, 18 hours) induced protein levels of IL6 (85.4%+/-9.8%) **(Fig. 1e)**. This inhibitory effect of 7α25HC was greater at reducing the mRNA levels of IL6 (53.3%+/-19.3%) **(Fig. 1f)**. Taken together, the data showed that EBI2 dampens pro-inflammatory signalling induced via different immune receptor signalling pathways such as TLR4 and TNFαR/IL17R in rodent and human astrocytes.

7α25HC inhibits IL17/TNFα-induced translocation of NFκB to the nucleus in human astrocytes

Oxysterols regulate levels of cytokines in immune cells via the NFκB signalling pathways (Englund et al., 2001; Lemaire et al., 1998; Leonarduzzi et al., 2005; Leonarduzzi et al., 2001; Ohlsson et al., 1996; Otaegui-Arrazola et al., 2010). To determine whether similar EBI2 signalling pathway exists in astrocytes, we treated human astrocytes with IL17/TNFα (50/10 ng/ml) with or without 7α25HC (1 μM) and stained for NFκB p65, while also counterstaining with Hoechst nuclei dye **(Fig. 2a, b)**. Treatment with IL17/TNFα induced translocation of NFκB p65 to the nucleus (218.9%+/-88.9%), in agreement with our previous study (Elain et al., 2014) **(Fig. 2b, c)**. In addition, 7α25HC alone had a moderate inhibitory effect on translocation of NFκB to the nucleus (65.1%+/-25.4%) possibly indicating tonic activity of EBI2, although this effect was not statistically significant **(Fig. 2b, c)**. Most importantly, the IL17/TNFα induced translocation of NFκB was significantly inhibited by 7α25HC (47.8%+/-25.6%) **(Fig. 2b, c)**. Taken together, these data suggest that EBI2 may exert inhibitory effects on IL17/TNFα-induced pro-inflammatory signalling, via a mechanism that involves inhibition of the NFκB signalling possibly. We provide a possible mechanistic pathway linking EBI2 signalling to cytokine release, that may include ERK1/2 and Ca^{2+} signalling, although we note this to be hypothetical and for illustrative purposes at present **(Fig 2d)** (Aye et al., 2012; Berridge, 2014; Dugas et al., 2010; Lemaire-Ewing et al., 2009; Rutkowska et al., 2015).

EBI2 agonism inhibits IL17/TNFα signalling in human astrocytes

To further investigate the role of EBI2 on IL17/TNFα signalling, using a different assay system, the effects of 7α25HC on cell impedance (CI) was measured using xCELLigence platform **(Fig. 3a)**. The data showed that IL17/TNFα (50/10 ng/ml) induced an increase in CI values, while the EBI2 agonist (7α25HC, 1 μM) and antagonist (NIBR189, 1 μM) did not induce significant changes **(Fig 3b)**. The IL17/TNFα increase in CI values, approximately 20 minutes following treatment, was significantly attenuated by pre-treatment with 7α25HC (295.3%+/-40.6% vs 231.1.%+/-38.1%) **(Fig. 3d)**. To demonstrate a direct EBI2 involvement, we first blocked EBI2 signalling with antagonist NIBR189 then treated with 7α25HC followed by IL17/TNF **(Fig. 3c, d)**. The data showed that NIBR189 partially blocked the inhibitory effects of 7α25HC (266.4%+/-35.9%) on IL17/TNF signalling increasing the CI values close to IL17/TNF levels **(Fig. 3d)**. These results further

demonstrated that human astrocytes signal in response to IL17/TNFα and that oxysterols 7α25HC significantly inhibits this response via the EBI2 receptor.

Enhanced pro-inflammatory cytokine release following LPS challenge in EBI2 KO mice

To demonstrate a direct EBI2 involvement in pro-inflammatory cytokine release *in vivo*, EBI2 KO mice were challenged for 4 consecutive days with LPS (0.5 mg/kg, i.p., qd) and pro-inflammatory cytokines were analysed **(Fig. 4a)**. The data showed that mice lacking EBI2 receptor had similar baseline cytokine profile as the WT controls **(Fig. 4b-e)**. In contrast, LPS challenge induced a significant increase in IL6 (199.3%+/-78.2%) **(Fig. 4b)**, TNFα (175.5%+/-60.9%) **(Fig. 4c)** and KCGRO (161.6%+/-27.2%) **(Fig. 4d)** cytokine levels in the EBI2 KO mice as compared to WT animals. An increase in IL1β levels was also observed (187.5%+/-92.9%) although significance (p=0.086) was not reached **(Fig.4e)**. This data again confirm a role for the EBI2 receptor in the regulation of pro-inflammatory response in the brains of these mice.

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DISCUSSION

We have recently demonstrated that EBI2 receptor, as well as the endogenous agonist 7α25HC and enzymes necessary for synthesis and degradation of 7α25HC, are expressed in human and mouse astrocytes (Rutkowska et al., 2016b; Rutkowska et al., 2015). We have also shown that 7α25HC induces pERK and Ca^{2+}intracellular signalling in astrocytes. In addition, we find that EBI2 induces astrocyte cell migration (Rutkowska et al., 2015) and that EBI2/oxysterol signalling is involved in the crosstalk between macrophages and astrocytes (Rutkowska et al., 2016b). Most recently we demonstrate that EBI2 regulates myelin development and inhibits LPC-induced demyelination. In the current study, we show that human, mouse and rat astrocytes release pro-inflammatory cytokines when stimulated with TNFα/IL17 or LPS, respectively and that pre-treatment with EBI2 agonist 7α25HC attenuates this response. We find that 7α25HC attenuates the TNFα/IL17-induced protein levels of IL6 in human astrocytes, via a mechanism that likely involves reduced levels of IL6 mRNA and via altered NFκB signalling. We also report that IL17/TNFα increases cell impedance in human astrocytes and that pre-treatment with 7α25HC significantly reduces this response. Moreover, we demonstrate that the EBI2 antagonist NIBR189 blocks 7α25HC-mediated attenuation of IL17/TNF-induced signalling, supporting a direct EBI2 involvement. Lastly, our *in vivo* LPS challenge study supports that EBI2 regulates pro-inflammatory cytokine signalling, whereby EBI2 KO mice exhibited enhanced pro-inflammatory cytokine release after LPS challenge as compared to WT mice. Taken together, these results demonstrate a role for EBI2/oxysterol signalling in limiting the levels of pro-inflammatory cytokines in the brain.

Data in the current study showed that pre-treatment of human astrocytes with 7α25HC inhibited both mRNA and protein levels of IL6 in TNFα/IL17-treated human astrocytes and furthermore that 7α25HC also reduced the protein levels of IL6 and TNFα in LPS-challenged mouse astrocytes. We also found that treatment of human astrocytes with 7α25HC attenuated the nuclear translocation of NFκB mediated by TNFα/IL17 treatment. In addition to regulating anti-inflammatory effects via NFκB, both pERK and Ca^{2+} signalling have been implicated in oxysterol-induced cytokine regulation (Aye et al., 2012; Dugas et al., 2010; Lemaire-Ewing et al., 2009). It may be relevant therefore that we have previously shown the EBI2 agonist 7α25HC also induces pERK and Ca^{2+} signalling (Rutkowska et al., 2015). Thus, in astrocytes, we suggest that EBI2 regulation of NFκB, pERK and Ca^{2+} signalling may play a role in altering cytokine signalling induced by TNFα/IL17 and LPS. In addition to

releasing pro-inflammatory cytokines, astrocytes may also respond to inflammatory triggers by changes in morphology, a likely prerequisite that allow astrocyte end-feet mediated regulation of the blood brain barrier and migrating to sites of inflammation. Using the xCELLigence impedance platform we showed that the EBI2 agonist, 7α25HC, decreased the TNFα/IL17-induced changes in cell impedance values, an effect reversed by pre-treatment with EBI2 antagonist NIBR189. These results further support the idea that EBI2 plays an important regulatory role in the response of astrocytes to inflammatory signals.

Our previous studies indicate that EBI2 is involved in the regulation of pro-inflammatory responses and inter-cellular communication under pathophysiological conditions such as LPS challenge (Rutkowska et al., 2016b). The data showed that media taken from LPS stimulated astrocytes induces macrophage migration (Rutkowska et al., 2016b). Moreover, other research has shown that mice deficient in CH25H enzyme cannot synthesise oxysterol 25HC and show aggravated course of EAE (Reboldi et al., 2014). In the same study, CH25H deficient mouse macrophages released greater amounts of IL1β after LPS stimulation than their wild type counterparts, suggesting that 25HC has anti-inflammatory properties (Reboldi et al., 2014). In line with these results, here the *in vivo* data showed that EBI2 KO mice challenged with LPS release greater amounts of pro-inflammatory cytokines such as IL6, TNFα and KCGRO than wild-type mice. These results support further a role for EBI2 in the regulation of pro-inflammatory cytokine signalling in the CNS.

Overall, this study support the hypothesis that EBI2/oxysterol signalling not only controls immune cell function, but may also adjust how astrocytes sense and respond to inflammatory stimuli.

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Disclosure/Conflict of interest

Andreas W. Sailer and Derya R. Shimshek are employees of Novartis Pharma, Basel, Switzerland

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FIGURE LEGENDS

Fig. 1. 7α25HC attenuates IL17/TNFα- and LPS- induced pro-inflammatory cytokines in astrocytes (a) Quantitative ELISA data shows that the EBI2 agonist 7α25HC (0.1 µM and 1 µM), does not induce IL6 release in rat astrocytes. Treatment with LPS (10 ng/ml or 100 ng/ml, 18 hours) induces significant increase in IL6 levels. Data presented as mean +/- SEM, one-way ANOVA and Bonferroni's post-test, ***p<0.001 vs. control. **(b)** 7α25HC inhibited LPS-induced IL6 release in a concentration dependent manner in rat astrocytes. Treatment with 7α25HC (1 µM and 0.1 µM, but not 0.01 µM) significantly inhibited IL6 release. Data presented as mean +/- SEM, one-way ANOVA and Sidak's post-hoc test, ***p<0.001, *p<0.05 vs. control. **(c)** Treatment of mouse astrocytes with 7α25HC (1 µM) attenuates LPS (100 ng/ml) mediated increase in the levels of IL6 and **(d)** TNFα protein levels. Data shown is a representation of three separate experiments (n=3). Data presented as mean +/- SEM, one-way ANOVA and Bonferroni's post-test, *p<0.05, ***p<0.001 vs. corresponding control. **(e)** Pre-treatment with EBI2 agonist 7α25HC (1 µM) attenuates IL17/TNF (50/10 ng/ml) mediated increase in the levels of IL6 protein and **(f)** mRNA in human astrocytes. Data shown is a representation of three separate experiments (n=3). Data presented as mean +/- SEM, one-way ANOVA and Bonferroni post-test, *p<0.05, **p<0.01 vs. corresponding control.

Fig. 2. 7α25HC attenuates IL17/TNFα-induced nuclear translocation of NFκB in human astrocytes (a) Schematic diagram illustrating experimental design **(b)** Representative confocal images show NFκB p65 (red) and nuclei (Hoechst, blue) immunostaining. **(c)** Bar graph shows 30 minutes pre-treatment with 7α25HC (1 µM) inhibited NFκB translocation to the nucleus induced by IL17/TNF (50/10 ng/ml, 18-20 hours) in human astrocytes. The number of Hoechst (blue) and NFκB (red) co-localising pixels were quantified by Zen 2011 software. Data presented as mean +/- SEM (n=4), one-way ANOVA and Bonferroni's post-test, *p<0.05, vs. corresponding control. Images taken at 40x magnification. Scale bar, 10 µm. **(d)** Tentative model showing potential mechanism of action of EBI2 inhibiting NFκB pro-inflammatory signalling: [1] activation of TNFα and IL17A receptors induces translocation of NFκB p65 (Berridge, 2014); [2,3] activation of EBI2 with 7α25HC induces Ca^{2+} signalling and phosphorylation of ERK1/2 (Berridge, 2014; Rutkowska et al., 2015); [4] this perhaps leads to activation of CREB that inhibits pro-inflammatory signalling (Berridge, 2014).

Fig. 3. 7α25HC inhibits IL17/TNFα-induced signalling in human astrocytes (a) Schematic diagram illustrating experimental design **(b)** Representative traces show effects of IL17/TNF (50/10 ng/ml), 7α25HC (1 µM) and NIBR189 (1 µM) on impedance cell index (CI) values over a time period of 36 minutes in human astrocytes. The IL17/TNF treatment induces marked changes on CI values, with little effect of NIBR189 or 7α25HC treatments alone versus non-treated control. **(c)** Representative traces show 30 minutes pre-treatment with 7α25HC (1 µM) inhibits IL17/TNFα (50/10 ng/ml) induced increase in CI values over a time period of 36 minutes in human astrocytes. Data presented as average (solid line) +/- SEM (dotted line) (n=4, observations) **(d)** A bar graph illustrates pre-treatment with 7α25HC (1 µM) significantly reduces IL17/TNFα (50/10 ng/ml) induced increase in CI values. Further pre-treatment with the EBI2 antagonist (NIBR189, 1µM) blocks the inhibitory effects of 7α25HC (1 µM) on IL17/TNF-induced CI values. Normalized cell index was calculated by subtracting the maximum response from the minimum response for each condition. Data presented as mean +/- SEM (n=8-12 observations), one-way ANOVA and Bonferroni's post-test, **p<0.01 vs. corresponding control.

Fig. 4. EBI2 KO mice release enhanced levels of pro-inflammatory cytokines following LPS challenge (a) Schematic diagram illustrating experimental design using EBI2 KO and WT mice in an LPS challenge paradigm. Quantitative MSD multiplex immunoassay technology shows that levels of pro-inflammatory cytokines in the brains of EBI2 KO mice treated with LPS (0.5 mg/ml, i.p., qd) or vehicle (PBS) for 4 consecutive days is significantly enhanced. Specifically, levels of **(b)** IL6 **(c)** TNFα and **(c)** KCGRO are increased. **(e)** Levels of IL1β in the brains of EBI2 KO mice also increased however, significance was not reached (p=0.086). Data presented as mean +/- SEM (n=4 animals for all treatment groups), one-way ANOVA and Tukey's post-test, *p<0.05, ***p<0.001 vs. WT LPS treated.

Supplemental Fig. 1. No observable microglial contamination of primary rodent astrocytes. Immunostaining of mouse astrocytes with astrocyte marker GFAP (red), microglia marker Iba1 (green) and nuclear stain Hoechst (blue) indicated that there was no detectable microglia presence in the primary mouse cell culture used for cytokine experiments.

Supplemental Fig. 2. Effects of LPS and IL17/TNFα on cytokine release in human and rodent astrocytes. Qualitative multiple cytokine array dot blots with accompanying key

show that LPS (100 ng/ml, 18 hours) treatment of does not significantly induce release of cytokines in **(a)** human astrocytes, but induces multiple cytokine release in **(c)** mouse and **(e)** rat astrocytes. Graphical densitometry of selected cytokines from dot blots shown in **(b)** human, **(d)** mouse and **(e)** rat astrocytes.

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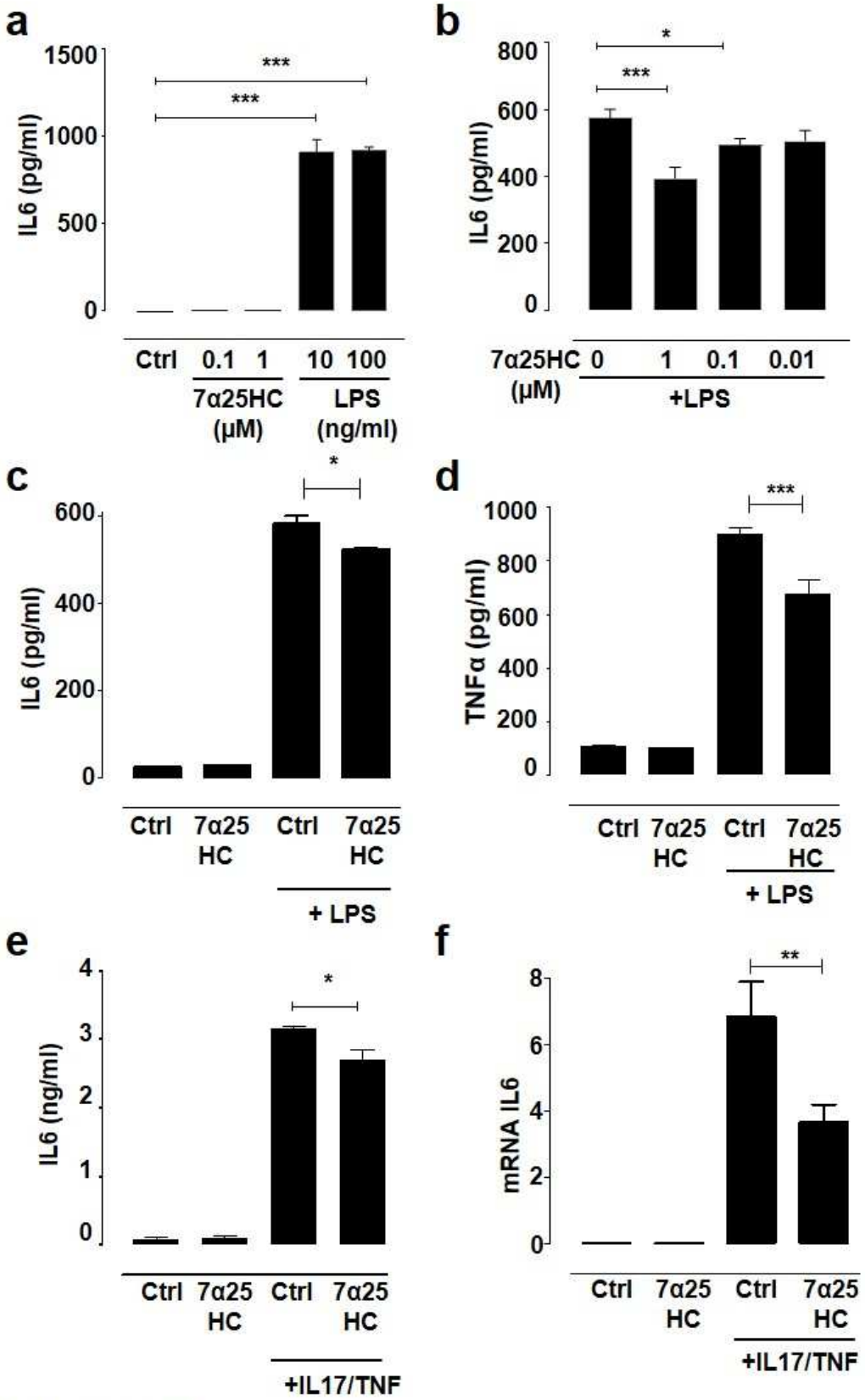
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Figure 1

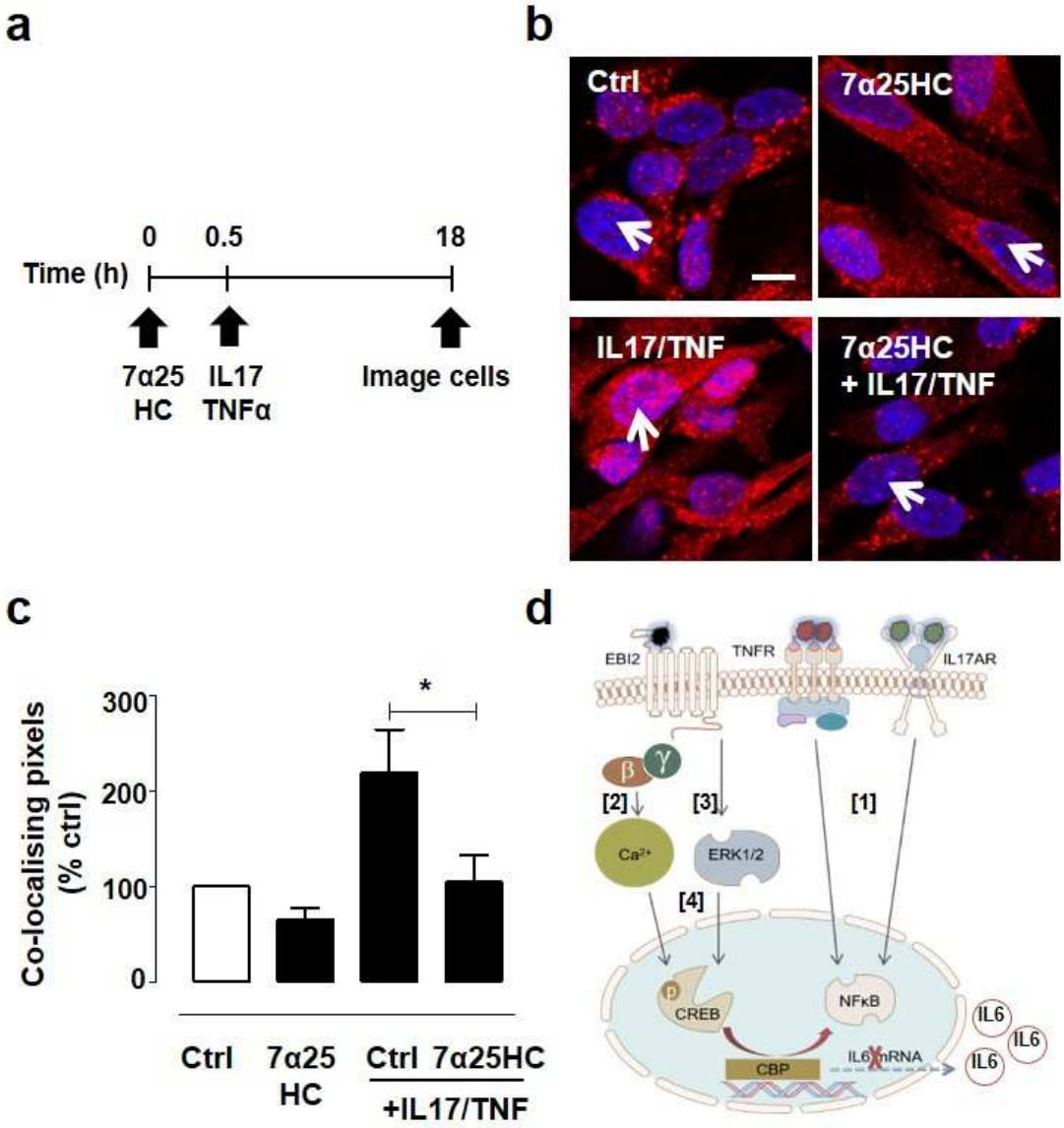
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Figure 2

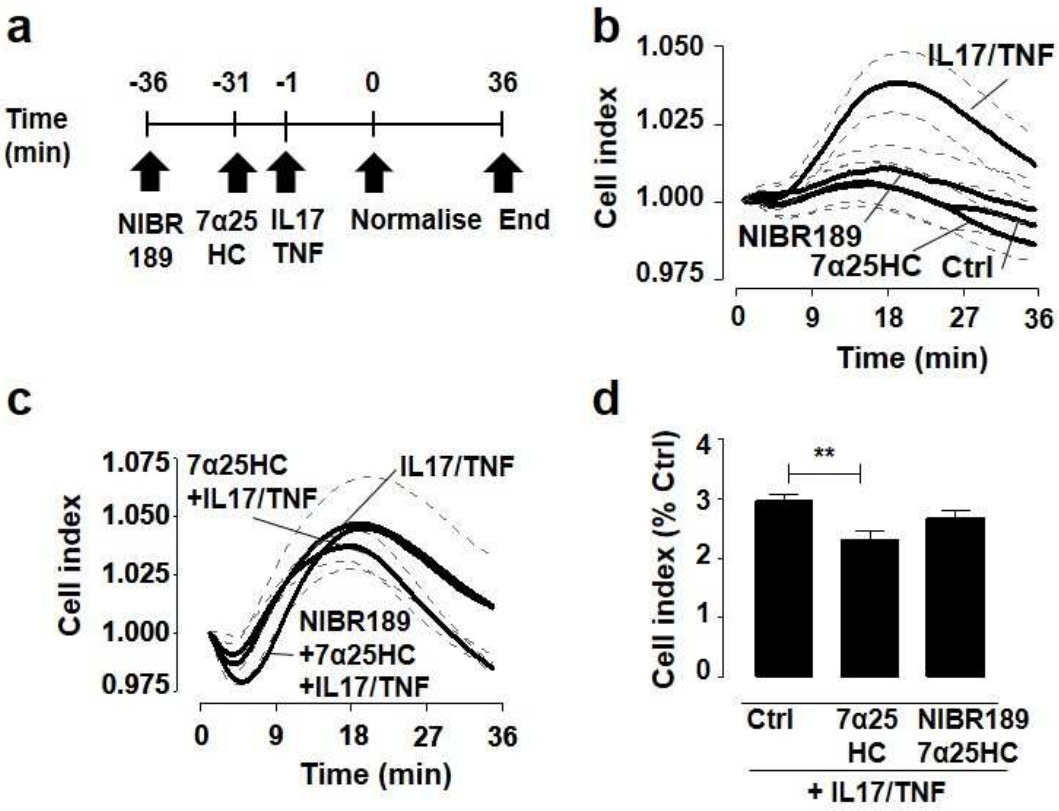

Figure 3

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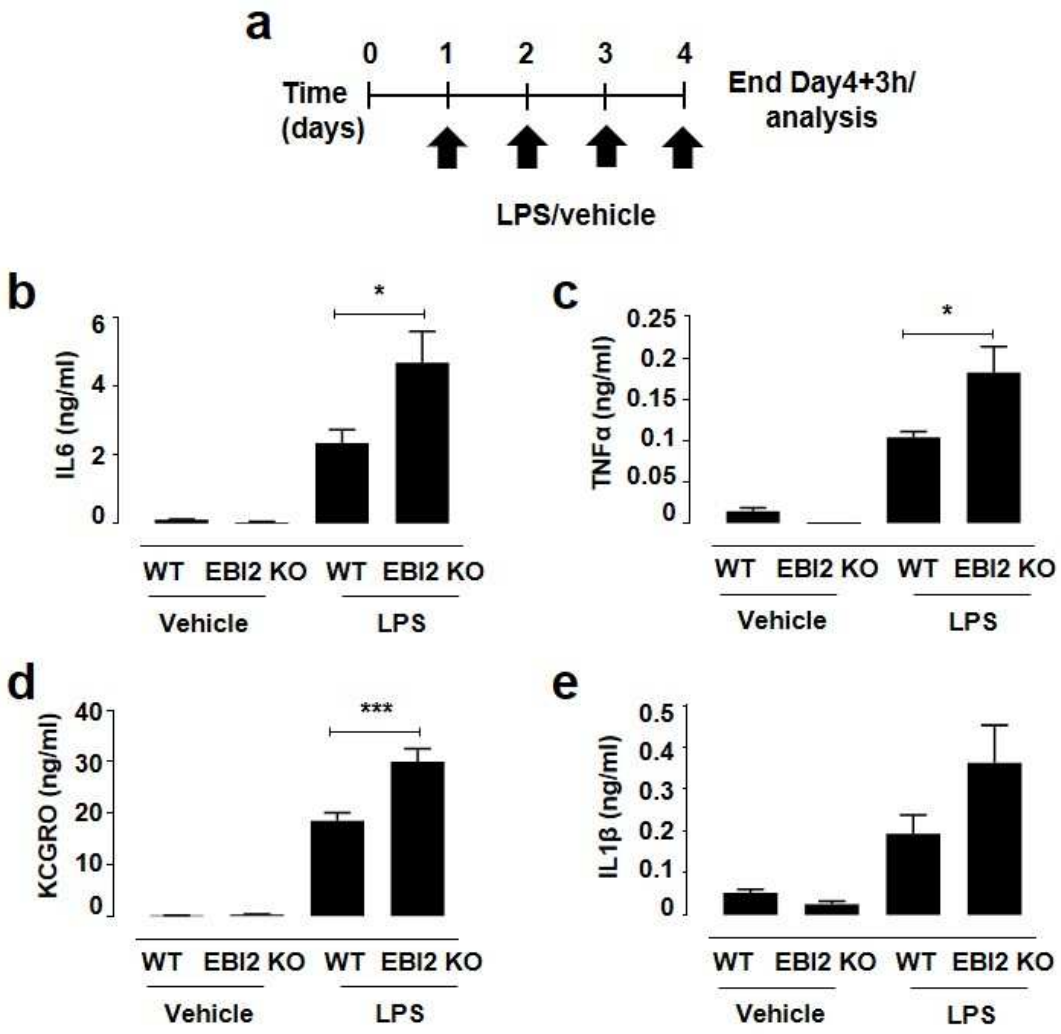

Figure 4

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Highlights:

- Oxysterol receptor EBI2 attenuates LPS- and IL17/TNF- induced pro-inflammatory cytokine release at protein and mRNA level in rodent and human astrocytes
- EBI2 receptor inhibits NFκB translocation to cell nucleus following pro-inflammatory IL17/TNF challenge
- Pre-activation of EBI2 receptor attenuates IL17/TNF-induced signalling in human astrocytes
- Knock-out of EBI2 receptor leads to enhanced pro-inflammatory cytokine release in LPS challenged mice
- Rodent and human primary astrocytes differentially respond to pro-inflammatory signals such as LPS or IL17/TNF

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