Ablation of type-1 IFN signalling in hematopoietic cells confers protection following traumatic brain injury

Type-1 IFN signalling regulates TBI outcome.

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Abstract

Type-1 interferons (IFNs) are pleiotropic cytokines, that signal through the type-1 IFN receptor (IFNAR1). Recent literature has implicated the type-1 IFNs in disorders of the central nervous system. In this study, we have investigated the role of type-1 IFNs in neuro-inflammation following traumatic brain injury (TBI).

Using a controlled cortical impact model, TBI was induced in 8-10 week-old male C57BL/6J WT and IFNAR1−/− mice and brains were excised to study infarct volume, inflammatory mediator release via qPCR analysis and immune cell profile via immunohistochemistry. IFNAR1−/− mice displayed smaller infarcts compared to WT mice after TBI. IFNAR1−/− mice exhibited an altered anti-inflammatory environment compared to WT mice, with significantly reduced levels of the pro-inflammatory mediators TNFα, IL-1β and IL-6, an up-regulation of the anti-inflammatory mediator IL-10 and an increased activation of resident and peripheral immune cells after TBI. WT mice injected intravenously with an anti-IFNAR1 blocking monoclonal antibody (MAR1) 1h before, 30 min after or 30min and 2d after TBI displayed significantly improved histological and behavioural outcome. Bone marrow chimeras demonstrated that the hematopoietic cells are a peripheral source of type-1 IFNs that drives neuro-inflammation and a worsened TBI outcome. Type-1 IFN mRNA levels were confirmed to be significantly altered in human post-mortem TBI brains. Taken together, these data demonstrate that type-1 IFN signalling is a critical pathway in the progression of neuro-inflammation and presents a viable therapeutic target for the treatment of TBI.
Significance Statement

This research article investigates the inflammatory effect of type-1 interferons (IFN) in traumatic brain injury (TBI), in both human and mice. IFNs have been traditionally associated with peripheral inflammatory responses. However, these molecules are also present in the central nervous system and we believe that they play a key role in the control of neuroinflammation. Our study shows that reducing type-1 IFN signalling, either by genetic ablation or by pharmacological intervention, has a beneficial effect on the outcome after TBI. IFN signalling is required for the brain to mount an inflammatory response to the insult of TBI. This study addresses key issues in type-1 IFN signalling and is a seminal discovery of their role in TBI.
Introduction

Traumatic brain injury (TBI) is the leading cause of death and disability in children and young adults in industrialised countries (Summers et al., 2009). There are currently no effective therapies clinically available to reduce the extent of tissue damage resulting from a TBI (McConeghy et al., 2012). TBIs are extremely heterogeneous with the extent of tissue damage highly dependent on the nature of the injury. However, a common feature of all TBIs is an initial primary lesion followed by a period of secondary tissue damage (Bramlett and Dietrich, 2004). The secondary damage seen in TBI results from complex morphological and biochemical changes in the brain that manifest following the initial impact and can last from days to weeks.

A prime player in the secondary expansion of tissue damage following TBI is neuro-inflammation (Greve and Zink, 2009). Neuro-inflammation is a complex phenomenon involving many different cell types and soluble factors and has been implicated as a common feature in many neuro-pathologies (Clausen et al., 2009; Pineau et al., 2010; Mikita et al., 2011; Wang et al., 2011). The involvement of astrocytes and resident microglia as well as peripherally invading cells adds to the complexity of TBI progression. A recent study investigating temporal neuro-pathological changes in humans following TBI found that neuro-inflammatory events continued for years after the initial injury and contributed to ongoing neuro-degeneration (Johnson et al., 2013). These ongoing neuro-pathological changes highlight the severity of the long-term consequences of neuro-inflammation following TBI. Therefore, a better understanding of the neuro-
inflammatory events elicited by TBI is needed to develop more effective therapies towards limiting tissue damage and degeneration.

Type-1 interferons (IFNs) are pleiotropic cytokines that are involved in responses to viral and microbial infections and cell proliferation (Pestka, 2007). Type-1 IFNs bind to and activate the IFNα receptor (IFNAR), which is comprised of type-1 interferon receptor 1 (IFNAR1) and IFNAR2 subunits (de Weerd et al., 2007). Engagement of this receptor complex leads to signalling through the canonical JAK-STAT pathway, resulting in the up-regulation of anti-viral and anti-proliferative proteins, including pro-inflammatory cytokines and chemokines and autocrine production of type-1 IFNs (IFNα and β) (Dai et al., 2011). Recent evidence indicates that injury to the CNS leads to up-regulation of type-1 IFN gene expression (Field et al., 2010). This finding suggests that type-1 IFNs may function as key mediators of the neuro-inflammatory response following TBI.

In this study, we investigated the role of type-1 IFN signalling in a controlled cortical impact (CCI) model of TBI. Using mice deficient in the IFNAR1 receptor subunit (IFNAR1−/−), we have demonstrated that blocking type-1 IFN signalling confers neuro-protection after TBI, and significantly alters the neuro-inflammatory milieu within the brain. Using a bone marrow chimeric approach, we have demonstrated that type-1 IFN signalling is involved in a deleterious role in hematopoietic cells to drive the neuro-inflammatory response following TBI. Furthermore, intravenous administration of a monoclonal antibody targeting IFNAR1 (MAR1) in mice is neuro-protective, with post-TBI administration
resulting in improved neurological function and decreased infarct size. Complementing this finding is data from human post-mortem brains following TBI that display increased type-1 IFN levels. In total, this data supports a detrimental role for type-1 IFN signalling following TBI, and also proposes that ablation of this signalling cascade promotes the development of an anti-inflammatory, neuro-protective environment within the brain.
Methods

Animals

All animal procedures were performed in accordance with the Author University animal care committee’s regulations. 8-10-week-old male mice (23±3 g) of a C57BL/6J background were obtained from the Animal Resource Centre. IFNAR1⁻/⁻ mice on a C57BL/6J background were previously generated at the Institute of Reproduction and Development at Monash University and were a kind gift from Professor Paul Hertzog (Hwang et al., 1995).

Controlled cortical impact model

Mice were anaesthetised with an intra-peritoneal injection of Ketamine (100mg/kg, Parnell)/Xylazine (10mg/kg, Parnell). A sagittal scalp incision was made to expose the underlying parietal skull. A 2 mm diameter plate of bone (centred 1.5 mm posterior to bregma and 2.5 mm lateral to the midline) was then removed using a Dremel 10.8V drill with a 0.8mm tip (Dremel, Europe) to expose the underlying right parietal cortex. A 1.5mm deep impact into the exposed cortex was made at 5m/s using the computer-controlled impactor device (LinMot-Talk 1100). Following impact, the bone plate was replaced and held in place with a small section of parafilm to cover the injury site. The skin incision was then closed with sterile silk 5.0 metric sutures (Syneture Tyco Healthcare). Mice were administered intra-peritoneal Buprenorphine (0.6 mg/kg, Reckitt Benckiser Healthcare) and placed on a heat mat for post-surgical recovery. Sham operated controls underwent the same anesthesia, scalp incision and bone plate removal, but were not injured.
MAR1 antibody administration

WT mice were intra-venously administered, in a blinded fashion, either a monoclonal antibody targeted toward IFNAR1 (Anti-mouse interferon α/β receptor [IFNAR1], Leinco Technologies Inc (MAR1), 0.5mg) or a monoclonal antibody isotype control (IgG isotype control, Leinco Technologies Inc, 0.5mg) either 1h prior to, or 30 min after TBI. In a separate cohort, WT mice were administered MAR1 or IgG both 30 min and 2d after TBI.

Behavioural analysis

Neurological function post-TBI was assessed using DigiGait™ v 11.5 (Mouse Specifics Inc.) apparatus, as previously described in (Sashindranath et al., 2012). Mice were run on a transparent treadmill at a speed of 15cm/s both before injury and 3h post-injury for 5s. Videos of paw placement were captured in the ventral plane by the DigiGait™ software and analysed by the software. All surgeries and behavioural analyses were performed in a blinded fashion. Gait measurements were calculated as post: pre injury ratios of sham versus trauma mice. All gait parameters for antibody-treated mice were presented as fold change relative to untreated TBI values.

Preparation of serial sections for staining and infarct analysis

Mice were trans-cardially perfused after injury (or sham surgery) with 0.1% heparinised Phosphate-Buffered Saline (Pfizer), followed by 4% paraformaldehyde (PFA, Scharlab S.L.). For infarct analysis, brains were collected 24h or 7d after TBI or sham surgery, paraffin-embedded, cut into 10μm
serial sections and mounted on glass slides (3 sections per slide). Every 10th slide was stained with Haematoxylin and Eosin (H&E). Images of the infarct were captured using an Olympus BX50 microscope fitted with a digital camera and infarct areas were measured using Image J software (NIH). The volume of tissue occupied by the infarct between successive pairs of serial sections across the infarct site was calculated from the area measurements in each section and the known distance between sections (300μm) and summed to determine total infarct volume (Figure 1).

For immunohistochemistry, brains were dissected after perfusion at 6 and 24h post injury or sham surgery and placed in 4% PFA for 2h and 10% sucrose (Univar) in PBS overnight at 4 °C. Brains were then placed into Optimal Cutting Temperature (OCT) medium (Tissue Tek) and frozen in Isopropanol on dry ice for a brief period and stored at -80°C until required. Brains were cut into 20μm fresh-frozen coronal serial sections using a Microm HM 525 cryostat.

**Immunohistochemistry**

Fresh frozen sections were incubated in 0.2% Triton X-100 (Sigma) in PBS for 20 min, and blocked for 30min in CAS block (Invitrogen). Primary antibodies were diluted in 1% BSA (Bovogen) in PBS and slides incubated with the antibody overnight at 4°C. The primary antibodies used were: CD206 (1:1000, BioScientific), Fox3 (1:200, Abcam), GFAP (1:1000, Cell Signaling), pSTAT3 (Ser727, 1:50, Santa Cruz) and Iba-1 (1:200, Wako). Slides were washed in PBS and incubated in an appropriate secondary antibody. Fluorescent secondary antibodies (Alexa Fluor 594 anti-mouse and Alexa Fluor 488 anti-rabbit;
Invitrogen) were used at 1:1000 (diluted in PBS). Sections were mounted in 
Vectashield with DAPI (Vector laboratories), and imaged under water immersion 
on a Leica DMI 6000B fluorescence microscope. Tiled images were imaged on a 
Zeiss Observer Z1 using Zen 2011 software. Quantification of GFAP and Iba-1 
staining was performed using Image J software (NIH), as described in (Baruch et 
al., 2014). The software generated fluorescence intensity values by tracing the 
region of interest (infarct in the ipsilateral hemisphere). Arbitrary units were 
defined in terms of strength of fluorescent signal. Tiled images were captured on 
the same day for all groups that were to be compared. In addition, fluorescence 
intensity analysis was done at the same time for groups that were to be 
compared. The final intensity values were calculated by subtracting the area of 
the selected region multiplied by the background fluorescence from the 
fluorescence intensity of the region of interest: Fluorescence intensity (arbitrary 
units) = Fluorescence intensity of R.O.I – (Area of selected region x Mean 
fluorescence of background).

Chimera development

C57BL/6 CD45.1 or IFNAR1−/− mice were irradiated (11 Gy in 2 equal doses, 2–3 
hours apart) to block hematopoietic cell production, as per (Downes et al., 2013) 
with the heads of the mice shielded. Recipient animals were then intravenously 
.injected with 1 x 10⁶ unfractionated bone marrow cells isolated from femurs of 
un-manipulated C57BL/6 CD45.1 or IFNAR1−/− donor mice. Percentage 
chimerism was determined 8 weeks after bone marrow transplantation using 
flow cytometry to assess levels of blood leukocytes using CD45-specific 
monoclonal antibodies. TBI surgeries were performed 3 weeks after assessing
levels of engraftment.

**Magnetic Resonance Imaging (MRI)**

MRI scans were performed for the chimera study using a Bruker 4.7 Tesla small animal MRI scanner (Florey Institute of Neuroscience and Mental Health) to quantify the progression of tissue damage, as described in (Crack et al., 2014). Mice were anesthetized with approximately 3% isoflurane in a 1:1 mixture of medical-grade air and oxygen. Anesthesia was maintained throughout scanning with 0.25 to 1.5% isoflurane through a nosecone placed over the animal’s snout and respiration was continuously monitored throughout the experiment with a pressure-sensitive probe positioned under the animal’s diaphragm. Anesthetized animals were laid supine on a purpose-built small-animal holder and their heads fixed into position with ear and bite bars. A surface receiver coil was placed over the animals’ heads and the cradle was inserted into a transmitter coil fixed inside a BGA12S-HP gradient set for imaging. The MRI protocol consisted of a 3-plane localizer sequence followed by multi-echo T2 and diffusion-weighted sequences. The total scanning time was kept to less than 2 h per animal. Multi-echo T2-weighted images were acquired using a rapid acquisition, relaxation enhanced (RARE) sequence with RARE factor = 2; repetition time = 2,500 ms; effective echo time (TEeff) = 10, 30, 50, 70, 90 and 110 ms; field of view (FOV) = 1.6 Å~1.6 cm²; matrix = 192 Å~192; and 16 slices with thickness = 0.5 mm. Volumetric analysis was carried out on T2-weighted images using ITK SNAP software [(Yushkevich et al., 2006); www.itksnap.org].

**Analysis of human samples by qPCR**
All procedures were conducted in accordance with the Australian National Health & Medical Research Council’s (NHMRC) National Statement on Ethical Conduct in Human Research (2007), the Victorian Human Tissue Act 1982, the Code of Ethical Autopsy Practice, and the Victorian Government Policies and Practices in Relation to Postmortem. Trauma brain samples from individuals who had died following closed head injury and non-head trauma controls were obtained from the Victorian Brain Bank Network (VBBN) (Frugier et al., 2009; Frugier et al., 2011; Frugier et al., 2012). Detailed patient is outlined in Table 1.

The following Taqman® primers for the human tissue samples were obtained from Applied Biosciences: IFNα (ID: Hs00256882_s1), IFNβ (ID: Hs01077958_s1), IFNAR1 (ID: Hs01066118_m1), IFNAR2 (ID: Hs01022060_m1), 18S ribosomal RNA (ID: Hs99999901_s1) and UBC (Hs01871556_s1).

**Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)**

Ipsilateral hemispheres were dissected 2, 4 and 24h after TBI or sham surgery, and RNA was isolated using Trizol® (Invitrogen). 1 μg of cDNA was transcribed from RNA using a high capacity cDNA reverse transcription kit (Applied Biosystems). Taqman® and SYBR® Green primers were obtained from Applied Biosciences and GeneWorks for mouse tissue samples. Ct values were obtained for each sample, and relative transcript levels for each gene were calculated using the 2-ΔΔCT method (Winer et al., 1999).

**Enzyme-linked Immunosorbent Assay (ELISA)**

Ipsilateral hemispheres were dissected after TBI or sham surgery. Tissue was
homogenised in Tris buffer (50mM Tris, 150mM NaCl, 1% Triton X-100, Phospho-STOP and Protease inhibitor [Roche]; pH 7.4) and rotated at 4°C for 90 min. Samples were centrifuged at 2000 x g and supernatant was collected. To determine protein concentration of samples, a Bradford assay was performed according to manufacturer's instructions (BioRad). Murine IL-1β, IL-6 and IL-10 ELISAs were purchased from BD Biosciences. 100μg of protein was loaded per well in duplicate. Protein concentrations of individual samples were determined using a linear curve of muIL-1β, muIL-6 and muIL-10 standards (4-250pg/mL).

Statistics

Data are expressed as Mean ± SEM, and analysed using Graph Pad Prism 5.0 software. Kolmogorov-Smirnov test (with Lilliefors correction) was used to test for normality within each group. For qPCR, ELISA and chimera infarct analysis, a one-way or two-way Analysis of Variance (ANOVA) was performed where appropriate followed by Dunnetts post-hoc analysis and Bonferroni’s post-hoc analysis, respectively. Infarct volume, fluorescence intensity values and Digigait™ behavioural data were analysed using an unpaired Student’s t-test. A value of p<0.05 was considered significant. Statistics summarised in table 2.
Results

TBI induces type-1 IFN signalling in mice

Firstly, we investigated the profile of IFN transcript regulation in ipsilateral hemispheres after controlled cortical impact in mice lacking the type-1 interferon receptor, IFNAR1. IFNα expression was significantly higher in WT mice 2h after TBI compared to IFNAR1-/- mice (p<0.001, Figure 2A). In addition, an up-regulation of IFNβ was seen 24h after TBI in WT, when compared to IFNAR1-/- mice (p<0.05, Figure 2A). This confirmed release of type-1 IFNs following TBI in mice. Signal transducer and activator of transcription (STAT) is a critical signal mediator in type-1 IFN signalling. STAT3 is phosphorylated or activated in this signalling cascade (Taylor et al., 2014), and here, pSTAT3 expression was used to assess the extent of type-1 IFN downstream signalling after TBI. pSTAT3 expression was assessed in neurons (stained with Fox3) and glia (stained with GFAP) in the ipsilateral hemisphere of both WT and IFNAR1-/- mice (Figure 2B and C). Fox3 was chosen as a neuronal marker due to its identification as the antigen for NeuN (neuronal nuclei) (Kim et al., 2009). 6h after TBI, pSTAT3 expression was elevated in neuronal cells in the cortex of WT mice compared to sham mice. In contrast, pSTAT3 expression could not be detected in corresponding IFNAR1-/- sections. However, pSTAT3 expression was detected in both the WT and IFNAR1-/- mice 24h after TBI and was co-localised with Fox3. Similarly, immunostaining identified an increase in pSTAT3 in WT mice 6h post TBI in glia, which was maintained 24h post TBI. IFNAR1-/- mice demonstrated no pSTAT3 staining 6h post TBI, but showed pSTAT3 immunoreactivity 24h post TBI, some of which co-localised with glia. This
suggests STAT3 may be activated through alternate pathways at later time points in the IFNAR1<sup>-/-</sup> after TBI. Additionally, we investigated interferon regulatory factor 7 (IRF7) mRNA levels following TBI. IRF7 is a key protein involved in type-1 IFN induction through various pathways, one of them being the type-1 IFN pathway itself (Marie et al., 1998; Sato et al., 1998; Honda et al., 2005). We identified an increase in IRF7 levels 2h post TBI in WT, but not IFNAR1<sup>-/-</sup> mice (p<0.01, Figure 2A). This further confirmed activation of downstream mediators of the type-1 IFN pathway in an IFNAR1-dependent manner.

**Mice lacking the IFNAR1 subunit have smaller infarct volumes after TBI**

The role of IFNAR in neuro-protection after TBI was of particular interest given we had established the involvement of type-1 IFN signalling in TBI in mice. To investigate this, WT and IFNAR1<sup>-/-</sup> mice were given TBI, and infarct volumes were measured in coronal H&E-stained brain sections taken 24h post-TBI. This experimental focal model of TBI produced a lesion confined to the cortical region of the ipsilateral hemisphere (Figure 3A). 24h after TBI, IFNAR1<sup>-/-</sup> mice had significantly smaller infarct volumes compared to their WT counterparts (3.52mm<sup>3</sup> compared to 6.96mm<sup>3</sup>, respectively; p=0.0047, n=6; Figure 3B).

**IFNAR1<sup>-/-</sup> mice display lower pro-inflammatory, and higher anti-inflammatory cytokine levels compared to WT mice after TBI**

To investigate the mechanism behind the neuro-protection seen in IFNAR1<sup>-/-</sup> mice, we investigated the expression profile of the pro-inflammatory genes IL-1β and IL-6 and the anti-inflammatory cytokine IL-10 in the ipsilateral hemispheres...
following TBI in both WT and IFNAR1-/- mice. mRNA levels of IL-1β were significantly elevated in WT, compared to IFNAR1-/- mice at 2, 4 and 24h after TBI (p<0.05 and p<0.001, n=3; Figure 4A). IL-6 was elevated in the ipsilateral hemisphere WT mice 4h after TBI compared to controls, but this up-regulation was not significantly different compared to the IFNAR1-/- mice at 4h (Figure 4B). IFNAR1-/- mice demonstrated increased IL-10 mRNA levels compared to WT mice both 2 and 4h after TBI (p<0.05, Figure 4C). To validate these data, we performed ELISAs to detect protein levels of the same cytokines. IL-1β and IL-6 protein levels were elevated in WT, compared to IFNAR1-/- mice at 6h (IL-1β) and 2h (IL-6; p<0.05, Figure 4D and 4E). IL-10 protein levels were elevated in IFNAR1-/-, compared to WT mice both 2 and 24h post TBI (p<0.01, Figure 4F).

These data imply signalling through IFNAR1 mediates the up-regulation of pro-inflammatory genes post-TBI. In addition, absence of IFNAR1 results in an increase in the anti-inflammatory cytokine, IL-10. This strongly suggests the preference for a heightened anti-inflammatory and suppressed pro-inflammatory response in these IFNAR1-/- mice.

IFNAR1-/- mice display an increase in GFAP and Iba-1 staining compared to WT mice

Astrogliosis is a common feature of neuro-inflammatory pathologies, and is characterised by an up-regulation in GFAP expression in astrocytes (Zamanian et al., 2012). This response may be either protective or deleterious in various pathologies (Terai et al., 2001; Paintlia et al., 2013). In WT and IFNAR1-/- mice subjected to TBI, an increase in GFAP staining was seen compared to sham-operated controls indicating a neuroinflammatory response elicited by the TBI
Interestingly, IFNAR1−/− mice displayed increased GFAP staining (Figure 5A) and expression compared to WT mice 24h after TBI (492.2 compared to 313.9 arbitrary fluorescence intensity units, respectively, p=0.001, n=5; Figure 5C). In addition, we performed immunohistochemistry to detect activated microglia and peripherally invading macrophages. Ionised calcium binding adapter molecule 1 (Iba-1) is expressed in both microglia and macrophages (Fukuda et al., 1996), and its expression is up-regulated by the activation of these cell types (microgliosis). Baseline Iba-1 levels were similar in WT and IFNAR1−/− shams (data not shown), and were increased in both WT and IFNAR1−/− mice following TBI compared to shams (Figure 6A). Additionally, we observed a trend for increased Iba-1 immunoreactivity in IFNAR1−/− mice compared to WT mice 24h after TBI (352.2 compared to 254.8 arbitrary fluorescence intensity units, respectively, p=0.053, Figure 6C). In the IFNAR1−/− mice we were expecting to see decreased immunoreactivity of both GFAP and Iba-1, however contrary to our hypothesis, we identified increased immunoreactivity of both astrocytes (GFAP) and microglia/macrophages (Iba-1) in IFNAR1−/− mice, suggestive of increased astro- and micro-gliosis. However, in conjunction with this increased GFAP and Iba-1 cellular response, we identified a decreased pro-inflammatory, and increased anti-inflammatory response (Figure 3). The M2 marker, CD206 was shown to have staining in the IFNAR1−/− brain after TBI when compared to the WT that is suggestive of an altered microglial phenotype (Figure 7). Collectively, these data support the presence of a dominant anti-inflammatory and potentially protective environment in the IFNAR1−/− mice following TBI.
MAR1, a monoclonal antibody targeted to IFNAR1, is effective at reducing infarct volume when administered before and after TBI in mice.

The neuro-protective effects of knocking out IFNAR1 were confirmed by administering an antibody to IFNAR1 (MAR1) to block the receptor either before or after injury. Pre-treatment with MAR1 1h before injury resulted in significantly smaller infarct volumes when compared to animals pre-treated with an IgG isotype control (5.12 mm³ compared to 8.37 mm³, p<0.0438, n=3; Figure 8A). Post-treatment with MAR1 30 min after injury also resulted in smaller infarct volumes compared to IgG isotype control-treated mice (5.77 mm³ compared to 9.23 mm³, p=0.0001, n=6; Figure 8B). Additionally, when mice were treated with IgG or MAR1 both 30min and 2d after TBI, MAR1-treated mice had smaller infarcts compared to IgG-treated mice 7d after TBI (8.50 mm³ compared to 14.11 mm³, p=0.035, n=8; Figure 8C). These results indicate that blocking type-1 IFN signalling is neuro-protective both over a short and long time course after TBI, highlighting the therapeutic potential of MAR1 for TBI treatment.

MAR1 administration results in decreased IFN levels and a dampened pro-inflammatory response

To investigate the mechanism of MAR1-elicited protection, we performed qPCR and ELISAs to detect levels of IFN and the pro-inflammatory cytokines IL-1β and IL-6. Again, we investigated changes in ipsilateral hemispheres of mice after TBI. IFNα mRNA levels were increased in IgG-treated mice compared to MAR1-treated mice 2h post TBI (p<0.001, Figure 9A). IFNβ mRNA levels were increased in IgG-treated mice compared to MAR1-treated mice 4h after TBI (p<0.05, Figure 9B). Levels of IL-1β and IL-6 were higher in IgG-treated mice compared to
MAR1-treated mice 4h post TBI, as measured by ELISA (p<0.05, Figure 9C and 9D), interestingly IL-10 levels were found to be unchanged by MAR1 treatment (Figure 9E). Similar to genetic ablation of IFNAR1, MAR1 administration suppressed the signalling of type-1 IFNs and pro-inflammatory cytokines following TBI.

Post-TBI administration of MAR1 significantly improves neurological function in injured mice

To identify changes in neurological function in mice following TBI, the Digigait™ system and software were used. Injured mice displayed impaired locomotor function in their left hind limb (contralateral limb to injury) compared to sham operated control mice in parameters such as stance/swing ratio, % swing in stride and % stance in stride 3h after TBI. Sham and TBI comparisons are presented as post:pre injury ratios in Figures 10A-C. Changes in these gait indices in the MAR1 antibody and IgG isotype-treated mice are presented as fold change relative to untreated WT TBI mice. We found that administration of MAR1 30 min post-TBI significantly improved locomotor function after TBI for the left hindlimb in the parameters of stance/swing ratio (Figure 10D, p=0.0124, n=10), % swing in stride (Figure 10E, p=0.0156, n=10) and % stance in stride (Figure 10F, p=0.0124, n=10). These results indicate that MAR1 is an effective treatment that improves neurological function and behavioural outcome after TBI.

Blocking type-1 IFN signalling in the hematopoietic cell compartment is protective following traumatic brain injury
In order to dissect the cellular mechanisms behind IFNAR1-mediated neuro-inflammation, we generated bone marrow chimeras of WT C57BL/6 CD45.1 and IFNAR1−/− mice. We performed TBI on 3 groups of mice: mice, which had IFNAR1 deleted, except in hematopoietic cells (WT→IFNAR1−/−), mice, which had IFNAR1 deleted only in hematopoietic cells (IFNAR1−/−→WT) and mice without IFNAR1 deletion (WT→WT). We assessed successful engraftment of donor cells using flow cytometry. All groups of mice demonstrated >95% engraftment in CD19 positive blood B cells and approximately 80% engraftment of CD19 negative T cells (data not shown). Engraftment levels were measured 8 weeks post transplantation. The level of engraftment in T cells reflects the fact that the T cells were largely resistant to the irradiation. WT radiation naïve mice displayed similar infarct volumes to WT mice reconstituted with WT bone marrow (data not shown). T2-weighted MRI demonstrated that the IFNAR1−/−→WT group had significantly lower infarct volumes 7d following injury, and displayed a trend for a reduced infarct volume 24h following injury (Figure 11A, C). In comparison there was no significant change in infarct volume in the WT→IFNAR1−/− group (Figure 11B, C). This finding reveals a critical role for type-1 IFN signalling in driving neuro-inflammation in peripheral hematopoietic cells following TBI.

Type-1 interferon signalling in the hematopoietic compartment influences astrogliosis and microgliosis.

An increase in GFAP and Iba-1 staining was observed in TBI mice compared to sham-operated chimeras (sham tiled images not shown). GFAP immunoreactivity was unchanged between all chimera groups 24h after injury when quantified as fluorescence intensity (data not shown). 7d post injury,
Type-1 interferons are involved in human TBI pathology

To investigate the contribution of type-1 IFN signalling in humans following TBI, we assessed type-1 IFN mRNA levels in post-mortem brains with qPCR (Figure 14). Details of human post-mortem tissue samples can be found in Table 1. A decrease in IFNα mRNA levels was identified in subjects that had died 3h after TBI (p=0.0019, Figure 14A). In contrast, IFNβ mRNA levels were significantly increased in subjects that had died 6h after TBI compared to controls (p=0.0001, Figure 14B). Levels of the receptor subunits IFNAR1 and IFNAR2 remained unchanged in injured brains compared to controls, indicating the potential for intact ligand-receptor interaction (Figure 14C). Type-1 IFNs are therefore...
implicated in humans after TBI, demonstrating the relevance of studying this system in neuro-inflammation following TBI.
Discussion

The current data show that ablation of IFN signalling through genetic deletion of IFNAR1 or pharmacological blockade of the receptor leads to pronounced protection after TBI. Previous studies have documented the roles of type-1 IFNs in responses to viral and tumour-associated pathologies (Hwang et al., 1995; Henry et al., 2007) but this is the first study to implicate type-1 IFN signalling in \textit{in vivo} acute neural injury. Type-1 IFNs are known to be released in response to cellular stress via toll-like receptor (TLR) pathways (Field et al., 2010), contributing to further damage and neuro-degeneration (Taylor et al., 2014). The role of type-1 IFNs in the CNS is an emerging field of study, with recent evidence suggesting that the type-1 IFN response contributes to the pathology seen in acute and chronic neuro-pathologies (Khorooshi and Owens, 2010; Wang et al., 2011; Taylor et al., 2014).

Our study highlights the involvement of type-1 IFN signalling in both mice and humans following TBI. We reported IFNAR1-dependent increases in IFNα and IFNβ after TBI in mice. In addition to primary type-1 IFN induction, it is known that type-1 IFNs are also produced through IFNAR signalling as a positive feedback mechanism (Gough et al., 2010). It is also possible that IFNAR drives the secondary production of these IFNs leading to the exacerbation of inflammation in humans. Interestingly, IFNα was elevated early in mice, contrasting to the down-regulation in IFNα mRNA in human TBI patients. In addition to this, IFNβ was elevated in humans to a greater extent than that seen in mice. The disparity between the human and murine results may be explained as the initiator of the neuro-inflammatory cascade may differ; being IFNβ in
humans and IFNα in mice. The production of IFNs is under tight regulation by IFN-producing pathways. It has been established that murine type-1 IFN release is controlled largely by the transcription factor IRF7 (Honda et al., 2005). A recent study in human blood monocytes demonstrated that IFNβ production was under joint control of the transcription factors IRF3 and IRF8 (Li et al., 2011). While these studies were conducted in models of infection, they do suggest that the regulation of type-1 IFN induction differs between mice and humans; hence the production of these cytokines will be influenced largely by which IRFs are dominant following infection or injury.

Downstream activation of type-1 IFN signalling was further confirmed after TBI with pSTAT3 immunohistochemistry and IRF7 induction. STAT3, a transcription factor, has broad roles in cell cycle regulation, and can be activated via IFN signalling pathways (Taylor et al., 2014). Recently, STAT3 phosphorylation was identified in astrocytes in a rat fluid percussion injury model of TBI where it was proposed that activation of STAT3 could contribute to inflammation or be neuroprotective depending on cell type (Oliva et al., 2011). In addition, STAT3 was found to be phosphorylated in an IFNAR1-dependent manner in a model of Alzheimer's disease, identifying STAT3 as a crucial downstream effector of type-1 IFN signalling (Taylor et al., 2014). In our CCI model, it was found that STAT3 was phosphorylated in GFAP-positive astrocytes and Fox3-positive neurons 6h following injury in an IFNAR1-dependent manner. This activation was absent in IFNAR1-/- brains. Our findings support a role for STAT3 as a critical downstream mediator of type-1 IFN signalling following CNS injury. In addition, IRF7 was induced 2h following TBI in WT, but not IFNAR1-/- mice. IRF7 is
implicated in type-1 IFN production and signalling, and it has been shown that absence of IRF7 impairs IFNα and β production (Honda et al., 2005). In a study of hippocampal sterile injury, type-1 IFN signalling pathways were activated via IRF7, leading to the release of downstream inflammatory mediators (Khoroooshi and Owens, 2010). Collectively, our results point to an engagement and activation of type-1 IFN signalling pathways following TBI.

Downstream of type-1 IFN pathway activation, we investigated the release of pro-inflammatory mediators IL-1β and IL-6. Type-1 IFN signalling influenced the release of these mediators, with diminished levels of IL-1β mRNA and both IL-1β and IL-6 protein in the IFNAR1−/− mice. The neutralisation of pro-inflammatory cytokines has often been associated with beneficial outcomes post-TBI (Clausen et al., 2009). Additionally, studies investigating therapeutics targeting inflammation post-TBI often report decreases in pro-inflammatory cytokine levels (Truettner et al., 2005; Lloyd et al., 2008). Taken together, this evidence proposes that suppression of the pro-inflammatory response in the IFNAR1−/− mice after TBI could explain a potential mechanism as to why these mice exhibit decreased lesion volumes.

Another crucial hallmark of the neuro-inflammatory cascade in TBI is the activation of resident astrocytes and microglia, and infiltration of peripheral immune cells (D’Mello et al., 2009; Pineau et al., 2010; Zamanian et al., 2012; Wang et al., 2013). Astrocytes are crucial in regulating responses to infection and neural injury, and respond to such challenges by becoming ‘reactive’ and up-regulating expression of glial fibrillary acidic protein (GFAP), in a process termed
Astrogliosis (Zamanian et al., 2012). Astrogliosis has been defined in the context of both neuro-degeneration and neuro-protection in the CNS, and reactive astrocytes are known to produce mediators such as pro- and anti-inflammatory cytokines and growth factors to elicit their effects onto the surrounding environment (Myer et al., 2006; Zamanian et al., 2012). Similarly, microglial cells can also undergo reactive gliosis under conditions of cellular stress or injury (Cao et al., 2012). In contrast to our results reporting decreased pro-inflammatory cytokines, we observed increased GFAP and Iba-1 staining in the IFNAR1⁻/⁻ mouse after TBI, indicative of increased astro- and microgliosis. Interestingly however, this increased gliosis in IFNAR1⁻/⁻ mice was observed concomitantly with diminished levels of pro-inflammatory mediators, and elevated levels of anti-inflammatory IL-10, suggestive of an 'M2-like' or reparative glial response to injury.

The Iba-1 antibody is known to detect both microglia and peripherally invading macrophages, therefore it is reasonable to expect the detection of both cell types in our TBI model. A recent study conducted in a mouse compression injury model of TBI demonstrated that an acute inflammatory response coordinated primarily by microglia, macrophages and neutrophils is neuro-protective and limits cell death within the meninges and deeper brain tissue (Roth et al., 2014). The increase in microglia and macrophages found in the IFNAR1⁻/⁻ mice following TBI may be eliciting a similar neuro-protective function. Similar to the astrocytes in the IFNAR1⁻/⁻ mice, activated microglia/macrophages in the absence of IFNAR1 may lack the ability to produce pro-inflammatory cytokines. This may explain why IFNAR1⁻/⁻ mice have a dramatically reduced cytokine load.
after TBI. These results highlight the possibility of distinctive differences in the neuro-inflammatory milieu between WT and IFNAR1⁻/⁻ mice after TBI.

Our results suggest that the IFNAR1⁻/⁻ mice demonstrate a greater M2-polarised environment compared to WT mice after TBI. This is highlighted by an up-regulation of IL-10 mRNA and protein levels in IFNAR1⁻/⁻ mice compared to WT mice after TBI. IL-10 is an anti-inflammatory cytokine, and is produced by immune cells undergoing a phenotypic switch from 'resting' to 'alternatively activated.' Consequently, IL-10 is used as an M2 marker (Wang et al., 2013). These alternately activated cells exhibit anti-inflammatory, pro-angiogenic features, and up-regulate various different cytokines and growth factors (Sica et al., 2006). In a mid cerebral artery (MCAO) model of stroke, IL-10 administration was found to suppress overexpression of pro-inflammatory mediators IFNγ and TNFα and reduce lesion volume (Liesz et al., 2009). Furthermore, IL-10 administration in a mouse model of TBI improved neurological recovery post-TBI, and was associated with reduced levels of TNFα and IL-1β expression in the cortex and hippocampus (Knoblach and Faden, 1998). These studies demonstrate that an increased anti-inflammatory environment via increased IL-10 can result in a shift towards suppression of a pro-inflammatory environment and consequent protection and may explain how increased IL-10 levels in the IFNAR1⁻/⁻ after TBI contributes to the resultant neuroprotection.

Additional to silencing type-1 IFN signalling genetically, we demonstrated protective effects of administering a blocking monoclonal antibody, MAR1. MAR1-treated WT mice demonstrated reduced infarct volumes, similar to
IFNAR1−/− mice. Furthermore, administration of MAR1 resulted in decreased production of the pro-inflammatory cytokines IL-1β and IL-6. Secondary production of the type-1 IFNs, IFNα and β, was also blocked by MAR1 administration. Finally, MAR1 treatment improved behavioural outcome in WT mice after TBI. Collectively, these results suggest that MAR1 treatment is effective at managing TBI-induced neuro-inflammation, leading to a beneficial post-traumatic outcome, and indicates its potential as a viable therapeutic for combating TBI. There is little doubt that in our model of TBI which has a disrupted blood brain barrier that the MAR-1 antibody is able to cross readily in to the brain. Whether this will occur with an intact barrier is unknown at this time. It is known that some monoclonal antibodies are able to enter the brain (Yu et al., 2011). What we do know from our bone marrow chimera experiments is that the hematopoietic compartment influences TBI outcome and IFNAR1 signalling is heavily implicated. This data suggests that it is possible that MAR1 does not need to cross the barrier, and it’s peripheral effects on type-1 IFN signaling contribute to neuroprotection in TBI. Future studies are underway to address this interesting mechanistic question.

To further investigate the cellular mechanisms leading to protection following TBI in the IFNAR1−/− mice, bone marrow chimeras were generated. Through the development of bone marrow chimeras, we ascertained that removal of type-1 IFN signalling in hematopoietic cells conferred protection after TBI. This finding proposes that hematopoietic cells are a large contributor to the deleterious aspects of type-1 IFN signalling after TBI. Many studies have utilised chimeric approaches to investigate the relative contribution of brain-derived and
These studies have implicated various inflammatory pathways, in both resident and peripheral immune cells after injury. The distinction between these two tissue compartments is an important factor to consider when trialling therapeutics for acute brain injuries. Furthermore, targeting novel neuroprotectants to cell types outside of the CNS may extend the window of therapeutic opportunity in the treatment of TBI.

We have clearly demonstrated a critical role for type-1 IFN signalling in TBI. Importantly, removal of type-1 IFN signalling, both genetically and pharmacologically confers protection after TBI. Detailed investigation into this response after TBI indicates that signalling in peripheral immune cells is crucial in driving the deleterious neuro-inflammatory cascade. Therefore, it is evident that specific targeting of the peripheral component of the type-1 IFN response represents a promising therapeutic option after brain injury.
Figure 1: Photomicrographs showing the full extent of the infarct in an 8-10 week-old C57BL6/J WT mouse. Images are of 10μm thick H&E-stained sections from a mouse perfused 24h after injury. Sections are labeled s10-s100. Damaged tissue is defined by a decrease in H&E staining intensity and an example of border demarcation is illustrated in these images. Scale bar represents 20μm.

Figure 2- TBI induces type-1 IFN release and downstream STAT3/IRF7 activation in an IFNAR1-dependent manner. A IFNα and IFNβ mRNA levels are elevated in ipsilateral hemispheres of WT, compared to IFNAR1−/− mice 2 and 24h following TBI, respectively. Data represent mean± SEM, n=3 per group, *p<0.05, ***p<0.001. B pSTAT3 immunoreactivity is observed 6h following TBI in WT, but not IFNAR1−/− neuronal cells (labelled with Fox3) in the ipsilateral hemispheres compared to sham-operated mice. pSTAT3 induction is demonstrated in both WT and IFNAR1−/− neurons 24h after TBI. C 6h after TBI, pSTAT3 expression is increased in WT, but not IFNAR1−/− mouse astrocytes (labelled with GFAP) in the ipsilateral hemispheres compared to sham-operated controls. pSTAT3 is expressed in both WT and IFNAR1−/− astrocytes 24h after TBI. Images are representative of n=3 independent experiments. Scale bar represents 50μm. D IRF7 mRNA levels are elevated in ipsilateral hemispheres of WT, compared to IFNAR1−/− mice 2h following TBI. Data represent mean± SEM, n=3 per group, **p<0.01.

Figure 3- Absence of IFNAR1 contributes to a smaller infarct volume in mice 24 hours after TBI. A Representative 10μm thick Haematoxylin and Eosin
(H&E) stained coronal brain section from a WT and IFNAR1−/− mouse given TBI. IFNAR1−/− mice have significantly reduced infarct volumes compared to WT mice 24h after TBI. Data represent mean ± SEM, *p<0.05, n=6 animals per group. Scale bar represents 2mm.

**Figure 4-** IFNAR1−/− mice display lower levels of pro-inflammatory cytokines and higher levels of the anti-inflammatory cytokine IL-10 in ipsilateral hemispheres compared to WT mice after TBI.  

A IL-1β mRNA levels are elevated at 2, 4 and 24h after TBI in WT, but not IFNAR1−/− mice.  
B IL-6 mRNA levels are elevated 4h after TBI in both WT and IFNAR1−/− mice.  
C IL-10 mRNA levels are elevated 2 and 4h after TBI in IFNAR1−/− compared to WT mice.  
D WT mice display elevated IL-1β protein compared to IFNAR1−/− mice 6h after TBI.  
E IL-6 protein levels are elevated in WT, compared to IFNAR1−/− mice 2h after TBI.  
F IFNAR1−/− mice display higher levels of IL-10 protein compared to WT mice 2 and 24h after TBI. Data represent mean ± SEM, n=3 per group, *p<0.05, **p<0.01, ***p<0.001.

**Figure 5-** IFNAR1−/− mice exhibit increased GFAP staining compared to WT mice after TBI.  

A Representative image of GFAP staining in the ipsilateral hemisphere of WT and IFNAR1−/− mice 24h after TBI. Scale bar represents 200μm.  
B High resolution image of GFAP staining in the ipsilateral hemisphere of WT and IFNAR1−/− mice 24h after TBI. Image region is outlined in the white box in panel A. Scale bar represents 50μm.  
C Quantification of GFAP staining in TBI mice, using fluorescence intensity values to quantify GFAP levels. Data represent mean ± SEM, n=5 per group, **p<0.01.
Figure 6 - Iba-1 levels increase after TBI, and are influenced by type-1 IFN signalling. A Representative image of Iba-1 staining in the ipsilateral hemisphere of WT and IFNAR1-/- mice 24h after TBI. Scale bar represents 200μm. B High resolution image of Iba-1 staining in the ipsilateral hemisphere of WT and IFNAR1-/- mice 24h after TBI. Image region is outlined in the white box in panel A. Scale bar represents 50μm. C Quantification of Iba-1 staining in TBI mice, using fluorescence intensity values to quantify Iba-1 levels. Data represent mean ± SEM, n=5 per group, p=0.0537.

Figure 7 – IFNAR1-/- microgila exhibit increased CD206 staining compared to WT mice after TBI. CD206 immunoreactivity is observed following TBI in IFNAR1-/- mice at a greater level compared to WT mice 24 hrs after TBI. CD206 immunoreactivity is co-labelled with the microglial marker Iba-1.

Figure 8- Pre- and post-treatment with MAR1 decreases infarct volume in WT mice given TBI both 24h and 7d after TBI. A WT mice were treated 1h before surgery with MAR1 (0.5mg) or an IgG isotype control (0.5mg); infarct was calculated 24h after TBI. Data represent mean ± SEM, *p<0.05, n=3 animals per group. B WT mice were treated 30 min after TBI with MAR1 or an IgG isotype control; infarct was calculated 24h after TBI. Data represent mean ± SEM, *p<0.05, n=6 animals per group. C WT mice were treated 30 min and 2d after TBI with MAR1 or an IgG isotype control; infarct was calculated 7d after TBI. Data represent mean ± SEM, *p<0.05, n=8 animals per group.
Figure 9- MAR1-treated mice display reduced type-1 IFN and pro-inflammatory cytokine secretion following TBI. A IFNα mRNA levels are elevated in IgG-treated mice, compared to MAR1-treated mice 2h after TBI. B IFNβ mRNA levels are elevated in IgG-treated mice, compared to MAR1-treated mice 4h after TBI. C IL-1β protein levels are elevated in IgG-treated mice, compared to MAR1-treated mice 4h after TBI. D IL-6 protein levels are elevated in IgG-treated mice, compared to MAR1-treated mice 4h after TBI. E IL-10 protein levels are unchanged by MAR1 treatment after TBI. Data represent mean ± SEM, n=3 per group, *p<0.05, ***p<0.001.

Figure 10- MAR1 post-treatment significantly improves behavioural outcome after TBI. 3h after surgery, TBI mice show significant behavioural impairment compared to sham mice in left hindlimb parameters such as stance/swing ratio, % swing in stride and % stance in stride (A-C). Data are presented as values of post:pre injury ratios. Post-TBI administration of MAR1 significantly improves behavioural outcome compared to IgG-treated mice in these parameters (D-F). MAR1 and IgG-treated values are presented as fold change to TBI. Data represent mean± SEM, *p<0.05, n=10 animals per group.

Figure 11- Blocking type-1 IFN signalling in hematopoietic cells engenders protection following TBI. A MRI T2 images from bone marrow chimeric mice showing TBI lesion 24h after injury. WT→WT mice represent C57BL/6 CD45.1 mice irradiated to abolish hematopoietic cells and reconstituted with BL/6 bone marrow. IFNAR1−/−→WT mice represent irradiated C57BL/6 CD45.1 mice reconstituted with IFNAR1−/− bone marrow. WT→IFNAR1−/− mice represent
irradiated IFNAR1-/- mice reconstituted with C57BL/6 CD45.1 bone marrow. Scale bar represents 1mm. B MRI T2 images from chimeric mice showing TBI lesion 7 days after injury. C IFNAR1-/-→WT mice have significantly reduced infarct volumes 7 days after injury. Data represent mean± SEM, **p<0.01, n=6-8 animals per group.

**Figure 12- GFAP immunoreactivity is significantly elevated in IFNAR1-/-→WT mice 7d after TBI.** A Representative tiled images using GFAP immunohistochemistry in chimera groups 7d after TBI. Scale bar represents 200μm. B High resolution image of GFAP staining in the ipsilateral hemisphere of all chimeras 7d after TBI. Image region is outlined in the white box in panel A. Scale bar represents 50μm. C Quantification of GFAP staining in TBI mice, using fluorescence intensity values to quantify GFAP levels. Data represent mean ± SEM, n=3 per group, *p=0.0273.

**Figure 13- Iba-1 immunoreactivity is significantly elevated in IFNAR1-/-→WT mice 7d after TBI.** A Representative tiled images using Iba-1 immunohistochemistry in chimera groups 7d after TBI. Scale bar represents 200μm. B High resolution image of Iba-1 staining in the ipsilateral hemisphere of all chimeras 7d after TBI. Image region is outlined in the white box in panel A. Scale bar represents 50μm. C Quantification of Iba-1 staining in TBI mice, using fluorescence intensity values to quantify Iba-1 levels. Data represent mean ± SEM, n=3 per group, **p=0.0047.
Figure 14- Type-1 IFN transcript levels are altered in humans after TBI, while receptor levels are unchanged. A IFNα mRNA levels are decreased compared to control in patients who died <3h after TBI. B IFNβ mRNA levels are elevated compared to control in patients who died >6h after TBI. C IFNAR1 and IFNAR2 mRNA levels are unchanged in post-mortem brains. Samples were taken from the ipsilateral hemisphere of these patients. Samples from the contralateral hemisphere of patients who died >6h after TBI are represented in the last bar (>6h CL). Data represent mean ± SEM, n=5-11 patients per group, *p<0.05.
References


Disclosure Statement

The authors declare that they have no financial or personal conflicts of interest that relate to this study.
C  GFAP fluorescence intensity  
24h post injury

![Graph showing fluorescence intensity comparison between WT and IFNAR1−/−.](image)
C. Iba-1 fluorescence intensity
24h post injury

![Graph showing fluorescence intensity comparison between WT and IFNAR1^-/-](image)

- WT: [Fluorescence intensity (arbitrary units)]
- IFNAR1^-/-: [Fluorescence intensity (arbitrary units)]

p = 0.0537
Behavioural changes after TBI

A  STANCE/SWING RATIO
  LEFT HINDLIMB

B  % SWING IN STRIDE
  LEFT HINDLIMB

C  % STANCE IN STRIDE
  LEFT HINDLIMB

Behavioural changes in MAR1- and IgG-treated mice

D  STANCE/SWING RATIO
  LEFT HINDLIMB

E  % SWING IN STRIDE
  LEFT HINDLIMB

F  % STANCE IN STRIDE
  LEFT HINDLIMB
Table 1: Details of trauma and non-trauma control cases.

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Controls

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Cases 1-10: cases with a survival time between 0 and 17 minutes; Cases 11-18: cases with a survival time between 30 minutes and 3 hours; Cases 19-27: cases with a survival time between 6 and 261 hours; Cases 28-37: control cases. All brains were obtained at autopsy. PMI, post mortem interval (time between death and brain retrieval); M, male; F, female.
Table 2. Summary of statistics from figures
Statistical analysis was performed using GraphPad Prism6.

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<td>p&lt;0.05</td>
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* Kolmogorov-Smirnov test (with Lilliefors correction) was used to test for normality within each group.