TGFβ receptor II gene deletion in leucocytes prevents cerebral vasculitis in bacterial meningitis

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In bacterial meningitis, chemokines lead to recruitment of polymorphonuclear leucocytes (PMN) into the CNS. At the site of infection in the subarachnoid space, PMN release reactive oxygen species, reactive nitrogen intermediates (RNI) and interleukin-1β (IL-1β). Although these immune factors assist in clearance of bacteria, they also result in neuronal injury associated with meningitis. Transforming growth factor beta (TGFβ) is a potent deactivator of PMN and macrophages since TGFβ suppresses the production of ROI, RNI and IL-1. Here, we report that the deletion of the TGFβ receptor II gene in PMN enhances PMN recruitment into the CNS of mice with *Streptococcus pneumoniae* meningitis. This was associated with more efficient clearance of bacteria, and almost complete prevention of intracerebral necrotizing vasculitis. Differences in PMN in the CNS of infected control mice and mice lacking TGFβ receptor II were not explained by altered expression of chemokines acting on PMN. Instead, TGFβ was found to impair the expression of L (leucocyte)-selectin on PMN from control mice but not from mice lacking TGFβ receptor II. L-Selectin is known to be essential for PMN recruitment in bacterial meningitis. We conclude that defective TGFβ signalling in PMN is beneficial in bacterial meningitis by ameliorating migration of PMN and bacterial clearance.

Keywords: innate immunity; stroke; neuronal injury; blood brain barrier; chemokines

Abbreviations: BBB = blood–brain barrier; BSA = bovine serum albumin; cfu = colony-forming units; ELISA = enzyme-linked immunosorbent assay; FACS = fluorescence-activated cell sorter; FITC = fluoroisothiocyanate; ICP = intracranial pressure; LPS = lipopolysaccharide; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; PEC = peritoneal exudate cells; PMN = polymorphonuclear leucocytes; RT–PCR = reverse transcription–polymerase chain reaction; TGFβ = transforming growth factor beta; TLRs = Toll-like receptors

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Introduction

Polymorphonuclear leucocytes (PMN) and macrophages have the capacity to phagocytose and kill bacteria. Binding of bacteria to pattern recognition receptors of the Toll-like receptor (TLRs) family on phagocytes leads to an inflammatory response. TLR2 and TLR4 recognize pneumococcal cell wall components and the pneumococcal cytotoxin pneumolysin, respectively (Yoshimura *et al.*, 1999; Malley *et al.*, 2003; Schroder *et al.*, 2003) In cooperation with other TLRs,

such as TLR1 (Schmeck *et al.*, 2006) downstream events—involving the MyD-88 adaptor protein, phosphorylation of interleukin receptor-associated kinase (IRAK), activation of tumour necrosis factor receptor-activated factor 6 (TRAF6), activating protein 1 (AP-1) and nuclear translocation of nuclear factor κB (NF-κB)—lead to the production of inflammatory cytokines and chemokines, including interleukin-1β (IL-1β) and tumour necrosis factor-α

(TNF-α) (for review, see Barton and Medzhitov, 2003; Akira et al., 2006). In pneumococcal meningitis, these cytokines together with reactive oxygen species, reactive nitrogen intermediates (RNI) and matrix metalloproteinases (MMP) contribute to the disruption of the blood–brain barrier (BBB), and thereby enable leucocytes to be recruited to the site of infection. Recruitment of PMN into the CNS depends on the expression of selectins and β2 integrins on both endothelial cells and PMN as well as on chemokines, particularly macrophage inflammatory protein-2 (MIP-2/CXCL2) and KC/ Groα (CXCL1) (for review, see Koedel et al., 2002; Meli et al., 2002). Owing to low concentrations of antibacterial antibodies and complement factors in the CNS, the elimination of bacteria at this site of the body is not efficient (Zwahlen et al., 1982). As a consequence, phagocytes are continuously stimulated, secrete inflammatory mediators and cause CNS damage. Disruption of the BBB leads to brain oedema formation and increased intracranial pressure (ICP) with impaired cerebral blood flow. These changes ultimately cause irreversible neuronal injury (for review, see Koedel et al., 2002; Nau and Bruck, 2002; Kim, 2003)

The delicate balance between leucocyte activation to cope with bacteria and leucocyte deactivation to prevent their harmful potential to cause tissue injury may be guided by transforming growth factor beta (TGFβ). There are three members of the TGFβ family—TGFβ1, TGFβ2, and TGFβ3—which share a high degree of homology both at the structural and functional level. In the CNS, TGFβ1 expression is largely confined to the meninges and choroid plexus, whereas TGFβ2 and TGFβ3 are expressed in neurons and glial cells (Flanders et al., 1991). They exert their effects through a heteromeric receptor complex consisting of type I and type II transmembrane serine/threonine kinase receptors. Upon ligand binding the type II receptor (TGFβRII) transphosphorylates and activates the type I receptor, which catalyses receptor-regulated SMAD transcription factor phosphorylation, and thereby in cooperation with co-SMADs enables TGFβ signalling (Massague, 1996; Yang et al., 2003). On peripheral blood monocytes TGFβ is chemotactic, enhances phagocytosis, activates the production of cytokines—IL-1, TNFα—and leads to increased expression of several integrin receptors including LFA-1, VLA-3 and VLA-5 (for review, see Letterio and Roberts, 1998). However, when testing TGFβ on tissue macrophages including microglia, the cytokine was found to inhibit phagocytosis as well as the production of TNFα, IL-1, IL-6, ROI, and to induce increased expression of IL-1 receptor antagonist (Tsunawaki et al., 1988; Turner et al., 1991; Suzumura et al., 1993; Kim et al., 2004).

The discrepancy between the macrophage activating and deactivating properties of TGFβ is also true for PMN. TGFβ exerts strong chemotactic responses on PMN in vitro and, upon intraarticular injection, induces synovial inflammation in mice (Wahl et al., 1987; Allen et al., 1990; Welch et al., 1990; Reibman et al., 1991). However, TGFβ was found to inhibit extravasation of PMN in thioglycollate-induced

peritonitis (Gresham et al., 1991). Experiments on the role of TGFβ in bacterial infections have resulted in conflicting results. In a murine model of autoimmunity, MRL/lpr mice have been reported to express constitutively high levels of TGFβ. Intravenous injections of anti-TGFβ antibodies improved the survival of MRL/lpr mice when infected intraperitoneally with Staphylococcus aureus or Escherichia coli; this effect of anti-TGFβ antibodies was associated with enhanced PMN extravasation to the site of infection (Lowrance et al., 1994). The data obtained in autoimmune MRL/lpr mice are in contrast with the ability of TGFβ to induce leucocyte recruitment and to improve microbial clearance when administered via intrabronchial routes to rats with E. coli pneumoniae (Cui et al., 2003). In early pneumococcal meningitis in rats, the effect of TGFβ on inflammation was dependent on the route of administration. The local, intracisternal administration of TGFβ was found to drive the inflammatory response (Koedel et al., 1996), whereas intraperitoneal application of TGFβ inhibited the same response (Pfister et al., 1992). Neither the role of endogenous TGFβ nor the role of TGFβ in more advanced meningitis models has been investigated so far. To address whether the endogenous production of TGFβ during bacterial infections affects the function of phagocytes, we have generated mice that lack TGFβ receptor II expression on PMN and macrophages (phag-TGFβRII$^{-/-}$ mice).

Material and methods
Generation of phag-TGFβRII$^{-/-}$ mice
TGFβRII$^{flox/flox}$ mice were obtained from Stefan Karlsson and Per Levéen, Lund University, Sweden (Leveen et al., 2002), and LysM-cre mice were generously provided by Irmgard Förster, University of Düsseldorf, Germany (Clausen et al., 1999). The TGFβRII$^{flox/flox}$ mice were crossed with LysM-cre to yield a homozygous TGFβRII$^{flox/flox}$ background and heterozygous background for LysM-cre. Cre non-expressing littermates were used as controls. The genotype of the offspring was determined by polymerase chain reaction (PCR) using the primer pair P3/P4 (P3: 5′-tatggactggctgctttttgtattc-3′ and P4: 5′-tggggatagaggtagaaagacata-3′) to distinguish floxed alleles (575 bp) from wild-type (422 bp) (Leveen et al., 2002). The primer combination Cre8/Mlys1/ Mlys2 (Cre8: 5′-cccagaaatgccagattacg-3′; Mlys1: 5′-cttgggctgccagaa-tttctc-3′; Mlys2: 5′-ttacagtcggccaggctgac-3′) results in LysM-cre amplicons of 700 and 1700 bp and in weight amplicons of 350 bp.

RNA was isolated from PMN by TRIzol (Invitrogen, Life Technologies) and reverse-transcribed by M-MuLV reverse transcriptase (Roche, Rotkreuz, Switzerland). For Taqman real-time PCR, the Applied Biosystems assays-on-demand for TGFβRII exon boundary 3/4 (Mm01348770_m1) and TGFβRII exon boundary 6/7 (Mm00436978_m1) were used. Detection of TGFβRII mRNA was performed by using the forward primer P1: 5′-acattactctgga-gacggtttg-3′ and the reverse primer P2: 5′-ggtagtgttcagcgagccatctt-3′. All real-time PCR reactions were performed and analysed on an ABI Prism 7700 Sequence Detection System™ (Perkin Elmer Applied Biosystems). The reactions for the target and the endogenous control (18s rRNA, Applied Biosystems,

No. 4310893) were performed in separate tubes, and the comparative C_T method was used for standardization.

Mouse meningitis model

The strain *Streptococcus pneumoniae* type 3, an isolate from the CSF of a patient with pneumococcal meningitis, was used in this study. Before use, the bacteria were subcultured on blood-agar plates, checked for purity, inoculated into brain–heart infusion broth (Oxoid Gmbh, Wesel, Germany), supplemented with 3% horse serum and 1% bovine albumin (Serva, Heidelberg, Germany) and incubated overnight at 35°C. Then, the broth was centrifuged and the sediment was washed and resuspended in phosphate-buffered saline (PBS). The final suspension was turbidometrically adjusted to a density of 0.5, thus achieving a concentration of 10^7 colony-forming units (cfu)/ml. For inactivation, the suspension was centrifuged again, and the pellet was incubated in 70% ethanol for 2 h on ice, washed twice and resuspended in PBS (to a concentration of 10^8 cfu/ml). The absence of viable *S. pneumoniae* was proven by the lack of colony formation in blood-agar plates.

Meningitis was induced by injection of 15 µl of a bacterial suspension containing 10^7 cfu/ml of *S. pneumoniae* type 3 into the cisterna magna under short-term anaesthesia with halothane. Twenty-four hours after infection, mice were evaluated clinically and treated with the antibiotic ceftriaxone (100 mg/kg intraperitoneally). The clinical status was evaluated before pneumococcal infection, as well as 24 and 48 h post-infection using a set of tasks, including a postural reflex test, a beam walk test, a body proprioception test and a spontaneous motor activity test. For postural reflex test, mice were lifted upon fixation of the tail and symmetry in the movement of the four limbs was examined. Score '0' indicates all four limbs extended symmetrically, 1 indicates limbs on one side extended to a lesser degree or more slowly than those on the other side, 2 indicates minimal movement of limbs on one or both sides and 3 indicates lack of movement of limbs on one or both sides. The goal of the beam walk task for a mouse was walking on wooden beams with decreasing diameters. The score was 0, 1 or 2, if a mouse was able to traverse a beam of 5, 9 or 13 mm in diameter, respectively. For failure of walking along the thickest beam whose diameter was 18 mm, the score assigned was 3. Body proprioception was tested by touching mice with a blunt stick on each side of the body. Score 0 indicates that mice reacted by turning head or were equally startled by the stimulus on both sides, 1 indicates that mice reacted slowly to stimulus on both sides and 2 indicates that mice did not respond to stimulus. For evaluation of spontaneous motor activity, mice were placed in the centre of a rectangular cage (30 cm length/20 cm width). Score 0 indicates that mice approached at least three walls of the cage within 60 s, 1 indicates that mice reached at least one wall within the test interval, 2 indicates that mice only barely moved without reaching a wall and 3 indicates that mice did not move. In addition, if mice showed seizures, tremor, pilo-erection or reduced vigilance, it scores 1 point for each parameter. Additional score points were given to mice that were hypothermic (1 point = body temperature was between 36 and 34°C; 2 points = body temperature was <34°C) and/or had substantial loss of weight (1 point = 6–12% loss of body weight; 2 points = >12% loss of body weight). The maximum clinical score was 19 and indicated severe disease, whereas a score of 0 was associated with healthy uninfected mice. Forty-eight hours after infection mice were again clinically evaluated and anaesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine.

Subsequently, a catheter was inserted into the cisterna magna to measure ICP and to determine CSF leucocyte counts. To measure bacterial titres, cerebella were dissected and homogenized in sterile saline. Cerebellar homogenates were diluted serially in sterile saline, plated on blood-agar plates and cultured for 24 h at 37°C with 5% CO_2. In supplemental experiments, phag-TGFβRII$^{-/-}$ and TGFβRII$^{flox/flox}$ mice were evaluated clinically 24 h after infection and followed by the determination of ICP, CSF leucocyte counts, bacterial titres and brain albumin concentrations.

Determination of blood–brain barrier integrity

To assess BBB integrity, mouse brain homogenates were examined for diffusion of albumin using enzyme-linked immunosorbent assay (ELISA) (ACRIS, Bad Nauheim, Germany), an abundant serum protein that is normally excluded from the brain by the intact BBB, using ELISA.

Analysis of cerebral bleeding and hydrocephalus

Mice brains were cut in a frontal plane into 10-µm-thick sections. Beginning from the anterior parts of the lateral ventricles, 9 serial sections were photographed with a digital camera in 0.3 mm intervals throughout the ventricle system. Haemorrhagic spots were counted and the bleeding area was measured.

Analysis of chemotaxis of PMN

Thioglycollate-elicited peritoneal exudate cells (PEC) were recovered 20 h (PMN) or 2 days (macrophages) after injection of 1 ml 3% Brewer thioglycollate medium (Sigma, Buchs, Switzerland). The peritoneal cavity was flushed with 10 ml of Hanks' balanced salt solution (HBSS) (wtihout Ca, Mg)/1% bovine serum albumin (BSA)/15 mM Ethylenediaminetetraacetic acid (EDTA); the cells were collected by centrifugation at 1200 r.p.m. and resuspended to 1×10^6 cells/ml X-Vivo 15 medium (BioWhittaker, Cambrex, Belgium)/2 mM glutamine. For fluorescence-activated cell sorter [fetal calf serum (FACS) sorting, PEC were resuspended in FACS buffer (2% FCS, 10 mM EDTA, in PBS)] and incubated with anti-mouse CD16/32 (Fc-block from BD Pharmingen, Basel, Switzerland) for 5 min, and then stained with anti-Gr-1 fluoro-isothiocyanate (FITC) (BD Pharmingen, Basel, Switzerland) and anti-mouse CD11b PE (BD Pharmingen, Basel, Switzerland) for 20 min. The Gr-1^{high}/CD11b$^+$ PMN were sorted by an FACStar Plus (Becton Dickinson). 7-Amino-actinomycin D (7-AAD) was used to exclude non-viable cells in flow cytometric analysis.

To assess chemotaxis of PMN mouse CXCL2 was diluted in X-Vivo 15 medium/2 mM glutamine at the indicated concentrations and transferred into the lower chamber of Transwell plates [Corning-Costar (3 µm pore size), Baar, Switzerland]. A total of 10^5 PMN were added to the insert in 100µl X-Vivo 15 medium/2 mM glutamine. The plates were incubated at 37°C and 5% CO_2 for 1 h. At the end of the incubation, the remaining cells on the upper membrane surface were carefully removed with a cotton swab. Migratory cells attached on the lower part of the filter were stained with DAPI (Molecular Probes, Netherlands) at 1 µM dilution for 20 min at 37°C and thereafter fixed in 4% paraformaldehyde in PBS. Migrated PMN were counted with a square graticule (Leica Microsystems Wetzlar GmbH, Germany), in five visual fields per filter (three horizontal and two vertical fields crossing the middle of the filter) at 200× magnitude.
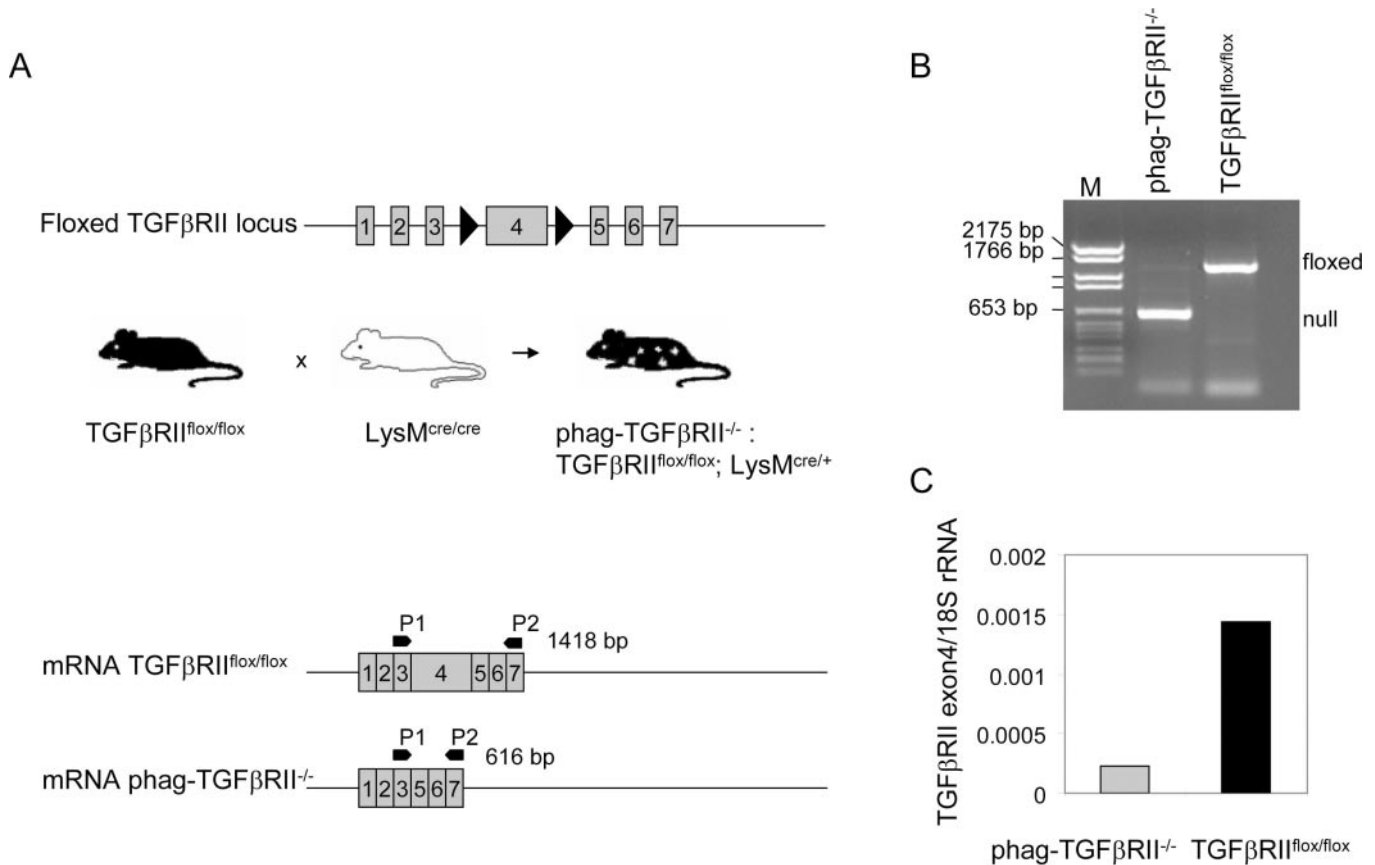
Fig. 1 Generation of mice with phagocyte-specific deletion of TGFβ receptor II. (**A**) Breeding scheme and detection of the deletion by RT–PCR. TGFβRII^flox/flox were crossed with Cre-bearing deleter mice in order to obtain phagocyte-specific TGFβRII deficiency. The indicated primers were used to verify the deletion of exon 4. (**B**) Deletion of exon 4 in total RNA of FACS-sorted Gr-1^high, CD11b^+ phag-TGFβRII^−/− PMN shown by RT–PCR analysis. (**C**) Deletion of TGFβRII exon 4 in phagocytes was determined by Taqman real-time PCR using primers specific for the boundary of exon 3 and 4 and normalized to 18S rRNA. Shown is one out of three representative experiments.

Flow cytometry

Brewer thioglycollate medium (1 ml), tuberculin purified protein derivative (PPD) (50 μg/animal in PBS, Statens Serum Institut, Denmark) or ethanol-inactivated *S. pneumoniae* were injected intraperitoneally into the right flank, and 10 h later, the mice were challenged with 1 μg of TGF-β (1 μg/0.5 ml PBS/1% BSA) or PBS/1% BSA into the left flank for an additional 10 h. PEC were recovered in HBSS (wtihout Ca, Mg)/1% BSA/15 mM EDTA and resuspended in FACS buffer. The cells were incubated with anti-mouse CD16/32 for 5 min and stained for 20 min at 4°C and washed. Antibodies used were as follows: FITC or PE-Gr-1, anti-mouse biotin-CD11b/APC-streptavidin, FITC-L-selectin or biotinylated-L-selectin/APC-Cy7-streptavidin (MEL-14) (BD Pharmingen) and PE-CXCR2 (R&D Systems, Oxon, United Kingdom). Immunofluorescence was detected by flow cytometry (Partec CyFlow, Münster, Germany). Data were analysed using WinMDI 2.8 software.

Detection of cytokines

Brains of mice were screened for 62 cytokines/chemokines using a commercially available mouse cytokine antibody array according to the manufacturer's instructions (RayBiotech, Atlanta, Georgia, Cat# M0309803). Additionally, CXCL2 and TGF-β were determined using commercially available ELISA kits (Quantikine Assay kits, R&D Systems GmbH, Wiesbaden-Nordenstadt, FRG). Briefly, frozen brain sections (with a total thickness of 1.8 mm) were homogenized in lysis buffer and then centrifuged at 12 000 r.p.m. for 15 min at 4°C, and 50 μl of the supernatant was used for each determination. Additionally, the protein concentration of the supernatant was measured using the Nanoquant assay (Carl Roth GmbH, Karlsruhe, FRG). Cytokine concentrations were expressed as picograms/milligram protein.

Determination of soluble L-selectin

The peritoneum was flushed with 10 ml of HBSS (w/out Ca, Mg)/1% BSA/15 mM EDTA and the soluble mouse L-selectin was measured by a commercially available ELISA kit (Quantikine). The values were obtained from three different experiments.

Production of TNFα by lipopolysaccharide (LPS) stimulated macrophages and microglia

PEC were seeded at a density of 5×10^5 cells/well in a 24-well tissue culture plate in DMEM/10% FCS/2 mM glutamine. Microglia were isolated as described before (Frei *et al.*, 1987). Briefly, each brain of
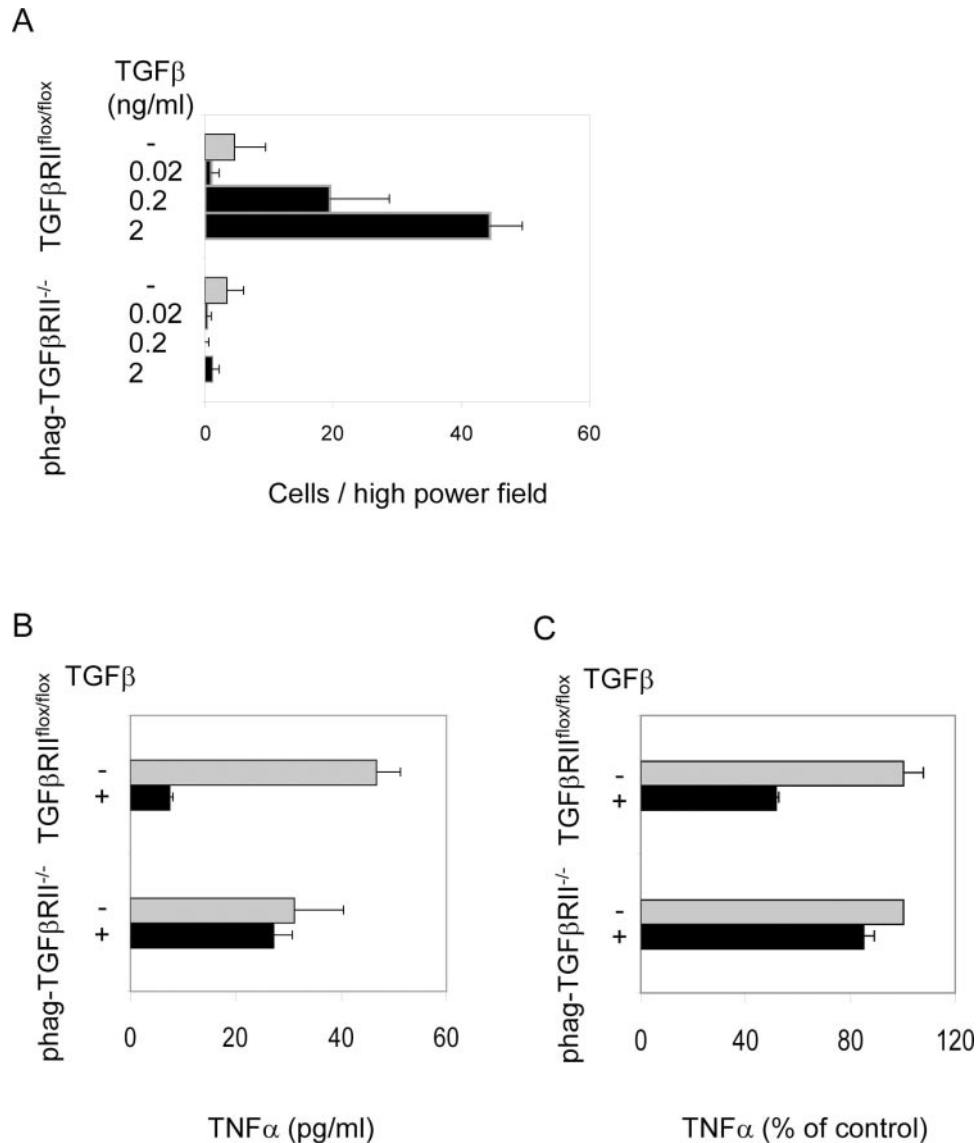
A

B

C

Fig. 2 TGFβ induces chemotaxis and inhibits TNFα production in leucocytes of TGFβRII^flox/flox mice but not in leucocytes of phag-TGF-βRII^−/− mice. (**A**) Migration of thioglycollate-elicited PMN of TGFβRII^flox/flox mice and phag-TGF-βRII^−/− mice in response to TGFβ (closed bars) or without TGFβ (grey bars). Human rTGFβ1 was added at various concentrations to the lower chamber of Transwell plates; PMN were added in the upper chamber and incubated for 1 h. Migrated cells were counted in five high power fields, and average values ± standard deviation are depicted. (**B**) Macrophages were pre-stimulated for 2 h with TGFβ1 (2 ng/ml) (closed bars) or without TGFβ1 (grey bars) followed by a 6 h incubation period with 0.01 ng LPS/ml. (**C**) Microglia were treated for 2 h with TGFβ1 (2 ng/ml) (closed bars) or without TGFβ1 (grey bars) followed by a 6 h incubation period with 1 ng LPS/ml. TNFα content in the supernatant was determined by ELISA.

newborn mice was cultured separately in a 75 cm^2 tissue culture flask for 14 days and the genotype was determined by PCR. By shaking, the microglia were separated from astrocytes and seeded at a density of 5×10^5 cells/well in a 24-well tissue culture plate in DMEM/10% FCS/2 mM glutamine. After 24 h, the medium was changed to X-Vivo 15/2 mM glutamine and macrophages or microglia were incubated overnight at 37°C, 5% CO$_2$. The cells were pre-stimulated with TGFβ1 (20 ng/ml) for 2 h and then 0.01 ng LPS/ml (055:B5; Sigma) was added for another 6 h. TNFα content in culture supernatants was measured by a TNFα ELISA Kit (BioSource Europe, Nivelles, Belgium).

Statistical analysis
The principal statistical test for analysing data obtained from *in vivo* experiments was one-way analysis of variance and Scheffe's test for *post hoc* analysis. Subset analysis of individual clinical parameters was performed, including spontaneous motor activity, beam balancing test and postural reflex test, using non-parametric Kruskal–Wallis test and Mann–Whitney *U*-test with alpha correction for *post hoc* comparison. The Spearman-rho correlation analysis was used to evaluate the relationship between meningitis-associated intracranial complications (like ICP or number of haemorrhage spots) or clinical outcome scores.
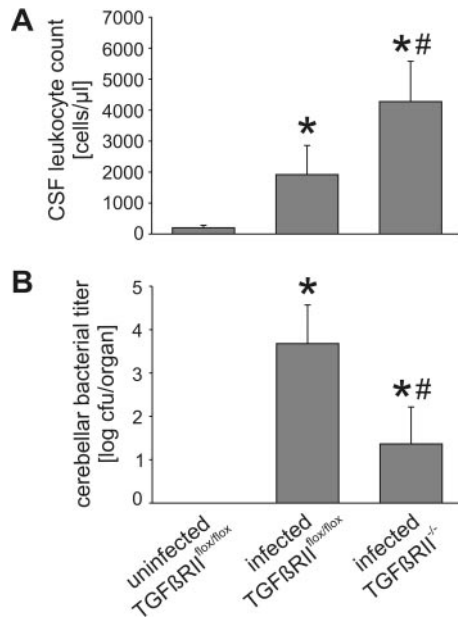
Fig. 3 High numbers of leucocytes in cerebrospinal fluid and low bacterial titres in the CNS of S. pneumoniae-infected phag-TGFβRII$^{-/-}$ mice. (**A**) As tested in phag-TGFβRII$^{-/-}$ mice (n = 14) and in TGFβRII$^{flox/flox}$ mice (n = 15), recruitment of leucocytes into the CSF of S. pneumoniae-infected mice treated with ceftriaxone is much more efficient for phag-TGFβRII$^{-/-}$ mice than for TGFβRII$^{flox/flox}$ mice (P < 0.001). (**B**) The higher CSF leucocyte numbers are associated with reduced cerebellar bacterial titres, indicating an improved clearance of bacteria in phag-TGFβRII$^{-/-}$ mice (P = 0.001). *Significant compared with ceftriaxone-treated TGFβRII$^{flox/flox}$; #significant compared with infected ceftriaxone-treated TGFβRII$^{flox/flox}$ mice using one-way analysis of variance and Scheffe's test for post hoc analysis. Data are expressed as means ± standard deviation.

Results
Generation of phagocyte-specific TGFβRII knockout mice

For the generation of mice that lack expression of TGFβRII on phagocytes, namely PMN and macrophages, TGFβRII$^{flox/flox}$ mice were crossed with Cre-bearing deleter mice (Fig. 1A). This mouse line expresses Cre under control of the murine lysozyme gene in macrophages and PMN (Clausen et al., 1999). As shown by reverse transcription–polymerase chain reaction (RT–PCR) analysis of total RNA, exon 4 is deleted in FACS-sorted Gr-1^{high}, CD11b$^+$ PMN from phag-TGFβRII$^{-/-}$ mice (Fig. 1B and C). Exon 4 encodes a majority of the TGFβRII kinase and the entire transmembrane domain. The amount of exon 6/7 detected by real-time RT–PCR in total RNA is similar in phag-TGFβRII$^{-/-}$ mice and TGFβRII$^{flox/flox}$ mice (0.00255 and 0.00253, respectively). In phag-TGFβRII$^{-/-}$ mice TGFβ-induced signalling is severely impaired in both PMN and macrophages. TGFβ induces a strong chemotactic response of Gr-1^{high}/CD11b$^+$ PMN isolated from the inflamed peritoneum of TGFβRII$^{flox/flox}$ mice. This contrasts with PMN from phag-TGFβRII$^{-/-}$ mice, which are completely resistant to TGFβ-induced chemotaxis

(Fig. 2A). These data confirm the capacity of TGFβ to exert a chemotactic response in PMN (Allen et al., 1990; Welch et al., 1990; Reibman et al., 1991). The failure of PMN from phag-TGFβRII$^{-/-}$ mice to migrate in response to TGFβ indicates that in thioglycollate-elicited PMN the lysozyme promoter is activated and thereby induces a deletion of the floxed TGFβRII gene. To test the efficiency of the lysozyme promoter to induce the TGFβRII deficiency in cells of the macrophage lineage, the inhibition of the production of TNFα by TGFβ was measured in LPS-stimulated macrophages and microglia. The LPS-induced secretion of TNFα was completely suppressed by TGFβ in cultured thioglycollate-elicited macrophages and to a lesser extent in microglia derived from the CNS of TGFβRII$^{flox/flox}$ mice. TGFβ did not impair TNFα production in macrophages and microglia cells obtained from phag-TGFβRII$^{-/-}$ mice (Fig. 2B and C). Taken collectively, these results show an almost complete unresponsiveness of PMN, macrophages and microglia cells to the functional effects of TGFβ and provide evidence that TGFβ is chemotactic for mouse PMN.

Improved innate immunity in S. pneumoniae- induced meningitis in phag-TGFβRII$^{-/-}$ mice

At 48 h after pneumococcal infection, brain concentrations of active TGFβ were significantly elevated in TGFβRII$^{flox/flox}$ mice (4.1 ± 2.1 pg/mg brain protein) compared with uninfected, PBS-injected control mice (1.1 ± 0.9 pg/mg brain protein; P = 0.020). To assess the functional role of the endogenous production of TGFβ on the host response to bacterial infection, we used mice that lack expression of TGFβRII on phagocytes. Infection of phag-TGFβRII$^{-/-}$ and TGFβRII$^{flox/flox}$ mice resulted in TGFβ concentrations of 1.8 ± 1.0 pg/mg brain protein and 4.1 ± 2.1 pg/mg brain protein (P = 0.027), respectively. Within 24 h after inoculation, all infected phag-TGFβRII$^{-/-}$ and TGFβRII$^{flox/flox}$ mice exhibited a similar degree of disease as evidenced by a loss of weight, hypothermia, pilo-erection, lethargy, as well as impaired motor activity and function. One out of 20 mice per strain died during the 24 h observation period. Moreover, at 24 h after infection, phag-TGFβRII$^{-/-}$ and TGFβRII$^{flox/flox}$ mice showed no differences in CSF leucocyte counts (7500 ± 4130 cells/μl versus 10 500 ± 5550 cells/μl, respectively), bacterial titres (8.7 ± 0.2 cfu/cerebellum versus 8.8 ± 0.4 cfu/cerebellum, respectively), rise in ICP and brain albumin concentrations (U. Koedel and H.W.Pfister, data not shown). Since, without antibiotic therapy, intrathecal challenge with 1.5 × 10^5 cfu S. pneumoniae causes death of all untreated mice within 45–48 h (U.Koedel and H.W. Pfister, unpublished data) (Gerber et al., 2001; Chiavolini et al., 2004), mice that were studied at a more advanced disease stage were treated with ceftriaxone given 24 h after pneumococcal inoculation. Twenty-four hours after the start of ceftriaxone therapy, phag-TGFβRII$^{-/-}$ mice exhibited significantly higher CSF leucocytes than TGFβRII$^{flox/flox}$
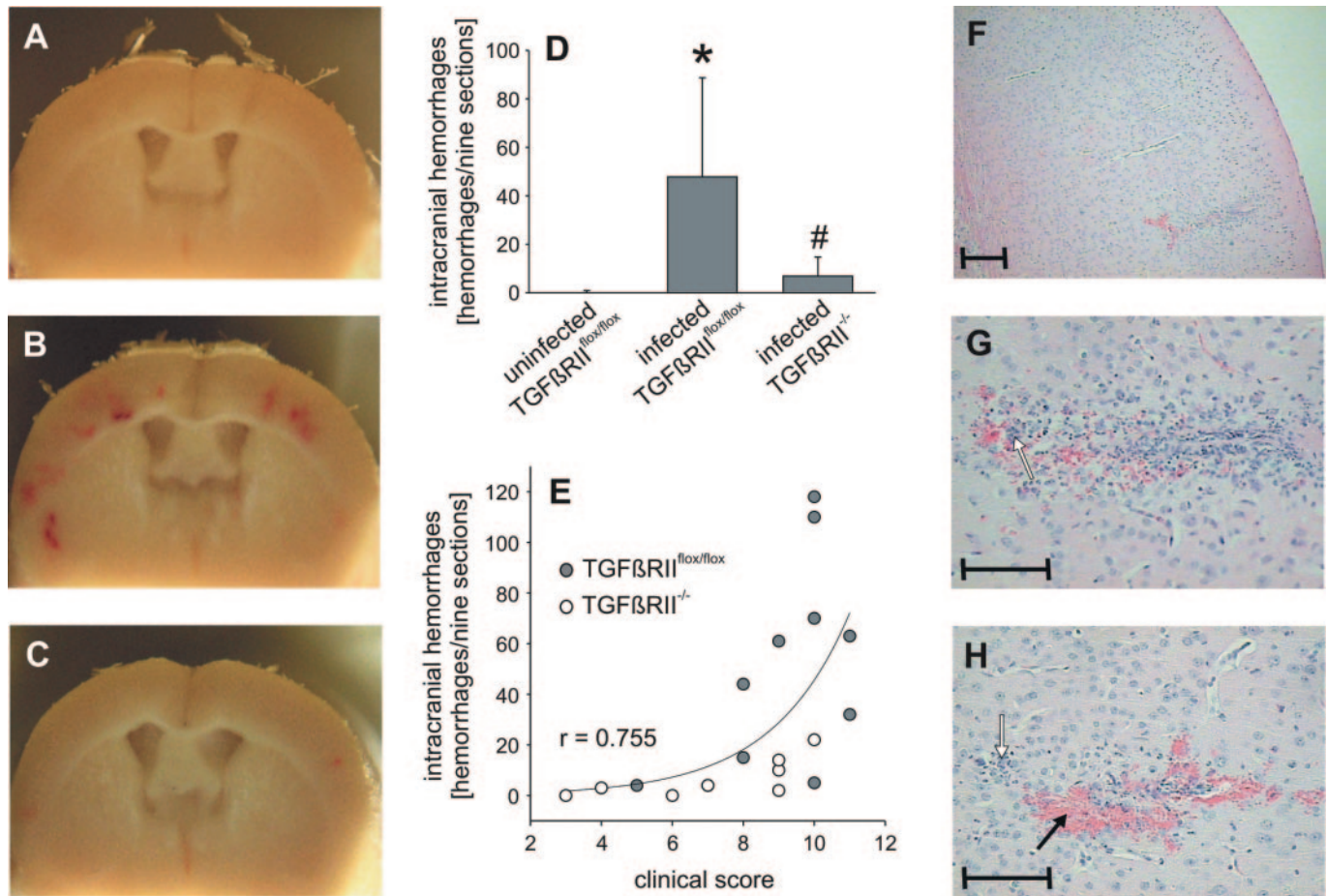
Fig. 4 Vasculitis and brain haemorrhages are a hallmark of *S. pneumoniae*-infected TGFβRII$^{flox/flox}$ mice but not of infected phag-TGFβRII$^{-/-}$ mice. Representative images of brain cryosections obtained from (**A**) an uninfected TGFβRII$^{flox/flox}$ mouse, (**B**) an infected TGFβRII$^{flox/flox}$ mouse and (**C**) an infected phag-TGFβRII$^{-/-}$ mouse. (**D**) In comparison with TGFβRII$^{flox/flox}$ mice, meningitis-induced intracerebral haemorrhages are only barely detectable in phag-TGFβRII$^{-/-}$ mice. Compared with the uninfected TGFβRII$^{flox/flox}$ controls, both infected mouse strains showed an enlargement of the lateral ventricles (hydrocephalus). (**E**) The number of intracerebral haemorrhages correlates with clinical symptoms of bacterial meningitis. (**F–H**) Histological analysis shows intraparenchymal vasculitis characterized by perivascular infiltration with PMN, nuclear dusts (open arrow) and destruction of the vessel wall with extensive haemorrhages in the parenchyma (closed arrow). Scale bar, 100 μm.

mice (Fig. 3A). The enhanced CSF pleocytosis was parallelled by a reduction of cerebellar bacterial titres, the latter being 140-fold lower in phag-TGFβRII$^{-/-}$ mice than in TGFβRII$^{flox/flox}$ controls (Fig. 3B). Thus, although TGFβ can promote prominent chemotaxis of PMN (Fig. 2A; Allen *et al.*, 1990; Welch *et al.*, 1990; Reibman *et al.*, 1991), there is no evidence that it plays such a role in bacterial meningitis.

Absence of vasculitis and intracerebral haemorrhages in phag-TGFβRII$^{-/-}$ mice

Since PMN are thought to play a pivotal role in the development of secondary brain damage in bacterial meningitis (Tauber *et al.*, 1988; Tuomanen *et al.*, 1989; Weber *et al.*, 1997), ICP, BBB integrity and brain pathology were assessed in both TGFβRII$^{flox/flox}$ mice and phag-TGFβRII$^{-/-}$ mice. The macroscopic hallmarks of the disease model used here were multifocal intracerebral haemorrhages (Fig. 4A, B and

C), which result from widespread leucocytoclastic vasculitis of small vessels in the brain cortex and less frequently in the white matter (Fig. 4F, G and H). Histological signs of disseminated intravascular coagulation were seen neither in TGFβRII$^{flox/flox}$ mice nor in phag-TGFβRII$^{-/-}$ mice. Likewise, infection with *S. pneumoniae* did not lead to bleeding disorders and coagulopathy since coagulation parameters (prothrombin time, activated partial thromboplastin time and fibrinogen degradation products) remained within the normal range. The plasma fibrinogen levels were even increased in both mouse strains (U. Koedel and H.W.Pfister, unpublished data). Interestingly, despite increased numbers of PMN in the CSF, phag-TGFβRII$^{-/-}$ mice had a 10-fold lower number of haemorrhages in the CNS than TGFβRII$^{flox/flox}$ mice (Fig. 4D). BBB damage as reflected by increased brain albumin concentrations was also significantly attenuated in the phag-TGFβRII$^{-/-}$ mice compared with TGFβRII$^{flox/flox}$ mice (Fig. 5B). As a consequence of the
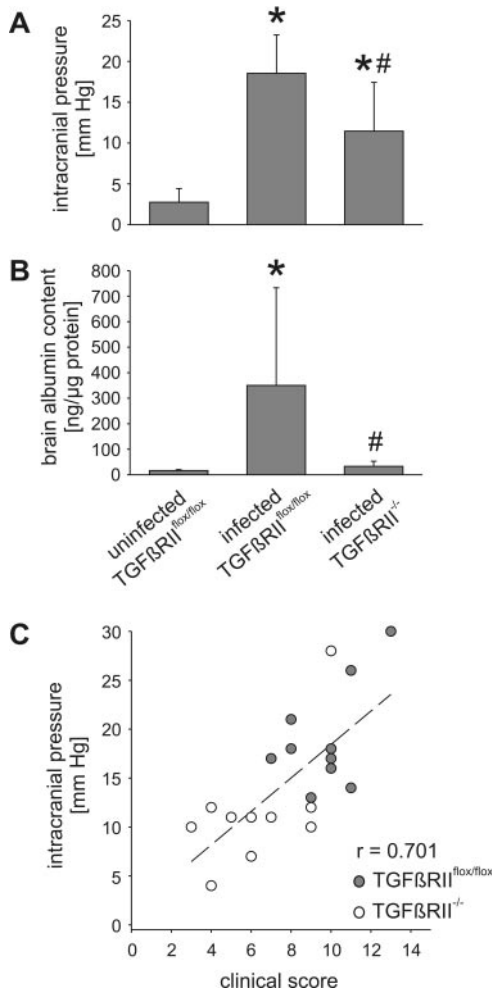
Fig. 5 Infected TGFβRII[flox/flox] mice have more pronounced secondary complications of meningitis than phag-TGFβRII^{−/−} mice. (**A**) At 48 h after infection, phag-TGFβRII^{−/−} mice had significantly lower ICP values than TGFβRII[flox/flox] mice. The *P*-value is 0.004. The statistical test analysis is done by one-way analysis of variance and Scheffe's test for *post hoc* analysis. (**B**) Vasogenic brain oedema as assessed by determination of brain albumin concentrations was significantly more pronounced in ceftriaxone-treated phag-TGFβRII[flox/flox] mice than in phag-TGFβRII^{−/−} mice (*P* = 0.042). (**C**) The increase of the ICP correlates with the clinical score of mice with bacterial meningitis. *Significant compared with ceftriaxone-treated TGFβRII[flox/flox]; #significant compared with infected ceftriaxone-treated TGFβRII[flox/flox] mice using one-way analysis of variance and Scheffe's test for *post hoc* analysis. Data are expressed as means ± standard deviation.

vascular lesions, both TGFβRII[flox/flox] and phag-TGFβRII^{−/−} mice showed a rise in the intracerebral pressure (ICP), which was also significantly less pronounced in phag-TGFβRII^{−/−} mice (Fig. 5A). The extent of intracerebral haemorrhages as well as the increase of the ICP correlated significantly with the clinical disease score, which was in turn significantly less affected in phag-TGFβRII^{−/−} mice compared with TGFβRII[flox/flox] mice (Figs 4E and 5C). Analysis of the individual parameters of the clinical score revealed significantly higher spontaneous motor activity and improved beam

balancing performance in phag-TGFβRII^{−/−} mice as compared with TGFβRII[flox/flox] mice; there were no significant differences in the other parameters investigated between both strains (Table 1). Taken collectively, the increase in the numbers of PMN in the CNS of phag-TGFβRII^{−/−} mice was associated with a decreased bacterial load and prevention of secondary brain damage, thus resulting in an amelioration of the clinical status.

TGFβ impairs the expression of ʟ(leucocyte)-selectin (CD62L) on neutrophils

Next, we assessed whether differences in expression of chemokines in phag-TGFβRII^{−/−} mice and TGFβRII[flox/flox] mice may be responsible for increased numbers of neutrophils in phag-TGFβRII^{−/−} mice. The increase of leucocytes in the CNS of phag-TGFβRII^{−/−} may be due to higher concentrations of chemokines that attract PMN, including CXCL1 (KC/GROα), CXCL2 (MIP-2) and CXCL5 (ENA/LIX). Protein arrays of mouse brain homogenates performed 48 h after infection showed CXCL2 but not CXCL1 and CXCL5 to be upregulated in infected TGFβRII[flox/flox] mice and phag-TGFβRII^{−/−} mice compared with uninfected control mice (U. Koedel and H.W.Pfister, data not shown). CXCL2 as well as CXCL1 have been identified to be responsible for chemoattraction of PMN in the CNF in mice with bacterial meningitis or meningoencephalitis (Seebach *et al.*, 1995; Diab *et al.*, 1999). When using ELISA techniques to quantify CXCL2 in *S. pneumoniae*-infected TGFβRII[flox/flox] mice and phag-TGFβRII^{−/−} mice, comparable concentrations were observed (7.0 ± 4.3 pg/mg brain protein in infected phag-TGFβRII^{−/−} mice versus 5.8 ± 3.6 pg/mg brain protein in infected TGFβRII[flox/flox] mice; the difference is not significant). These data do not support the possibility that major differences in expression of chemokines that induce chemotaxis of PMN may account for different numbers of PMN in the infected CSF of TGFβRII[flox/flox] mice and phag-TGFβRII^{−/−} mice.

Since the number of PMN that can be harvested from the CNS of mice with meningitis is too small to allow their characterization, PMN were studied in the peritoneal exudates of mice injected with thioglycollate, an effective inducer of neutrophil-mediated inflammation (Lewinsohn *et al.*, 1987). This approach is also given by the recent finding that TGFβ inhibits neutrophil migration in thioglycollate-induced peritonitis (Gresham *et al.*, 1991). Thioglycollate was injected into the peritoneal cavity of TGFβRII[flox/flox] mice and phag-TGFβRII^{−/−} mice; after 10 h either TGFβ (1 μg per mouse) or control solutions were injected intraperitoneally and PMN were harvested from the peritoneal exudates 10 h later. The effect of TGFβ on the expression of CXCR2, the receptor of the chemokine CXCL2 guiding PMN chemotaxis in bacterial meningitis, was measured on PMN by flow cytometry (Fig. 6A). TGFβ altered the expression of CXCR2 neither in TGFβRII[flox/flox] nor in phag-TGFβRII^{−/−} mice (Fig. 6A). However, in TGFβRII[flox/flox]

Table 1 Clinical status of mice 48 h after induction of pneumococcal meningitis

Experimental group (n)	Total clinical score	Motor activity[a]	Postural reflex text[b]	Beam walk test[c]	Body proprio-ception[d]	Presence of seizures	Presence of tremor	Presence of pilo-erection	Reduced vigilance
PBS-injected controls (4)	0.0	0.0	0.0	0.0	0.0	0 of 4 (0%)	0 of 4 (0%)	0 of 4 (0%)	0 of 4 (0%)
Infected TGFβRII^flox/flox (14)	9.2 ± 2.0[e]	1.3 ± 0.4	1.1 ± 0.9	1.9 ± 0.8	0.4 ± 0.5	0 of 14 (0%)	2 of 14 (14%)	6 of 14 (43%)	10 of 14 (71%)
Infected TGFβRII^−/− (13)	6.6 ± 2.3*	0.7 ± 0.4*	0.7 ± 0.8	0.7 ± 0.7*	0.5 ± 0.5	0 of 12 (0%)	1 of 12 (8%)	5 of 12 (42%)	7 of 12 (58%)

[a]Spontaneous motor activity was tested by placing mice in the centre of rectangular cage (30 cm length/20 cm width). Score 0 indicates that mice approached at least three walls of the cage within 60 s, 1 indicates that mice reached at least one wall within the test interval, 2 indicates that mice only barely moved without reaching a wall and 3 indicates that mice did not move. [b]For postural reflex test, mice were lifted upon fixation of the tail and symmetry in the movement of the four limbs was examined. Score 0 indicates all four limbs extended symmetrically, 1 indicates limbs on left side extended to a lesser degree or more slowly than those on the right, 2 indicates minimal movement of left-side limbs and 3 indicates lack of movement of left-side limbs. [c]The goal of the beam walk test for a mouse was walking on wooden beams with decreasing diameters. The score was 0, 1 or 2, if a mouse was able to traverse a beam of 5, 9 or 13 mm in diameter, respectively. For failure of walking along the thickest beam whose diameter was 18 mm, the score assigned was 3. [d]Body proprioception was tested by touching mice with a blunt stick on each side of the body. Score 0 indicates that mice reacted by turning head or were equally startled by the stimulus on both sides, 1 indicates that mice reacted slowly to stimulus on both sides and 2 indicates that mice did not respond to stimulus. [e]Data are means ± standard deviations. *P < 0.01, compared with infected TGFβRII^flox/flox using Kruskal–Wallis and Mann–Whitney U–test plus alpha corrections for post hoc analysis.

mice TGFβ is found here to reduce the expression of L (leucocyte)-selectin (CD62L) on PMN by 60% as calculated from two different experiments. This effect was dependent on TGFβRII since L-selectin was not different in PMN derived from TGFβ-treated or untreated phag-TGFβRII^−/− mice (Fig. 6B). In addition, tuberculin PPD and inactivated *S. pneumoniae* were used to induce PMN influx into the peritoneum. TGFβ treatment in TGFβRII^flox/flox mice impaired L-selectin expression in both conditions. However, no change of L-selectin expression was observed in TGFβ-treated phag-TGFβRII^−/− mice (Fig. 6C and D). TGFβ treatment did not affect the amount of shedded L-selectin measured by ELISA in the peritoneal lavage of thioglycollate-induced mice (with TGFβ 3.9 ± 1.5 ng/10^6 cells and without TGFβ 4.3 ± 1.5 ng/10^6 cells in TGFβRII^flox/flox mice; with TGFβ 5.2 ± 0.8 ng/10^6 cells and without TGFβ 3.8 ± 1.0 ng/10^6 cells in phag-TGFβRII^−/− mice). These results indicate that TGFβ interferes with the expression of L-selectin, a major adhesion receptor that has been shown to allow leucocyte rolling, which is a precondition for firm leucocyte adhesion to vascular endothelium and migration of PMN in meningitis (Lewinsohn *et al.*, 1987; Arbones *et al.*, 1994; Granert *et al.*, 1994; Tedder *et al.*, 1995).

Discussion

We have disrupted the TGFβ receptor II gene in phagocytes and found that the absence of TGFβ signalling facilitates the recruitment of PMN and the clearance of *S. pneumoniae* in the CNS of mice with meningitis. The findings in phag-TGFβRII^−/− mice reported here are in agreement with previous data from our group that (i) the administration of TGFβ dampens meningeal inflammation in early pneumococcal meningitis at which time endogenous levels of TGFβ in the CNS are still unaltered (Pfister *et al.*, 1992) and that (ii) anti-TGFβ antibodies improve the antibacterial host response in MRL/lpr mice infected with *E. coli* or *S. aureus* (Lowrance *et al.*, 1994). The effect of anti-TGFβ antibodies was only tested in autoimmune MRL/lpr mice, but not in congenic MRL control mice, which, in contrast to the MRL/lpr strain, do not express increased levels of active TGFβ in peritoneal exudates after infection with *S. aureus* (Lowrance *et al.*, 1994). However, our findings in phag-TGFβRII^−/− mice are in conflict with (i) the potent chemotactic response of TGFβ on PMN *in vitro* (Fig. 2; Reibman *et al.*, 1991) and (ii) the induction of inflammation when injecting TGFβ into the joints (Allen *et al.*, 1990; Fava *et al.*, 1991; Seebach *et al.*, 1995), with the observation that neutrophils from mice that lack the TGFβ-induced SMAD3 transcription factor fail to follow a chemotactic gradient formed by TGFβ *in vitro* or *in vivo* when injecting TGFβ subcutaneously (Yang *et al.*, 1999). Both bacterial meningitis and the inflamed peritoneum, the disease models showing that TGFβ reduces neutrophil recruitment, comprise inflammatory responses leading to the activation of leucocytes and endothelial cells, expression of adhesion molecules and production of

proinflammatory cytokines. This contrasts the experimental paradigms used to show chemotactic effects of TGFβ, namely either the injection of TGFβ into the non-inflamed joint or skin or the use of TGFβ in chemotactic chambers. The data presented here provide evidence that PMN lacking TGFβRII show a much better extravasation into the inflamed CNS in the course of the innate immune response to *S. pneumoniae* in experimental meningitis when compared with PMN with intact TGFβ receptor-mediated signalling.

To define the mechanism of impaired recruitment of PMN to sites of inflammation, PMN were analysed for their expression of chemokine receptors and adhesion molecules. PMN harvested from the thioglycollate-induced inflamed peritoneum of TGFβ-treated TGFβRII$^{flox/flox}$ and phag-TGFβRII$^{-/-}$ mice did not differ in their expression of CXCR2, the receptor which is required for the recruitment of neutrophils in experimental bacterial meningitis (Seebach *et al.*, 1995). However, a striking feature of TGFβ-treated PMN of TGFβRII$^{flox/flox}$ mice is their profound reduction

of the expression of L-selectin, an adhesion molecule that plays an essential role in migration of PMN to sites of inflammation (Lewinsohn *et al.*, 1987; Granert *et al.*, 1994). Inhibition of L-selectin expression by TGFβ was observed not only in thioglycollate-elicited PMN but also when inducing PMN by intraperitoneal injection of PPD or inactivated *S. pneumoniae*. Compared with control mice L-selectin-deficient mice show a significant reduction of PMN in thioglycollate-induced peritoneal exudates (Arbones *et al.*, 1994; Tedder *et al.*, 1995). Similar results were obtained in normal mice that have been injected with L-selectin neutralizing antibodies (Watson *et al.*, 1991). Furthermore, extravasation of granulocytes was also diminished in joints of L-selectin-deficient mice with experimental autoimmune arthritis (Szanto *et al.*, 2004). In the context of the data shown in the present study, it is of importance that fucoidin, which blocks the function of L-selectin, reduces the accumulation of PMN and plasma proteins in the CSF of rabbits with meningitis induced by intrathecal injections of *S. pneumoniae* antigens (Granert *et al.*, 1994; Angstwurm *et al.*, 1995; Brandt *et al.*, 2005). On the basis of the reported functions of L-selectin our data suggest that defective TGFβ signalling in PMN improves PMN recruitment into the CNS by increasing L-selectin expression on PMN. However, the deletion of
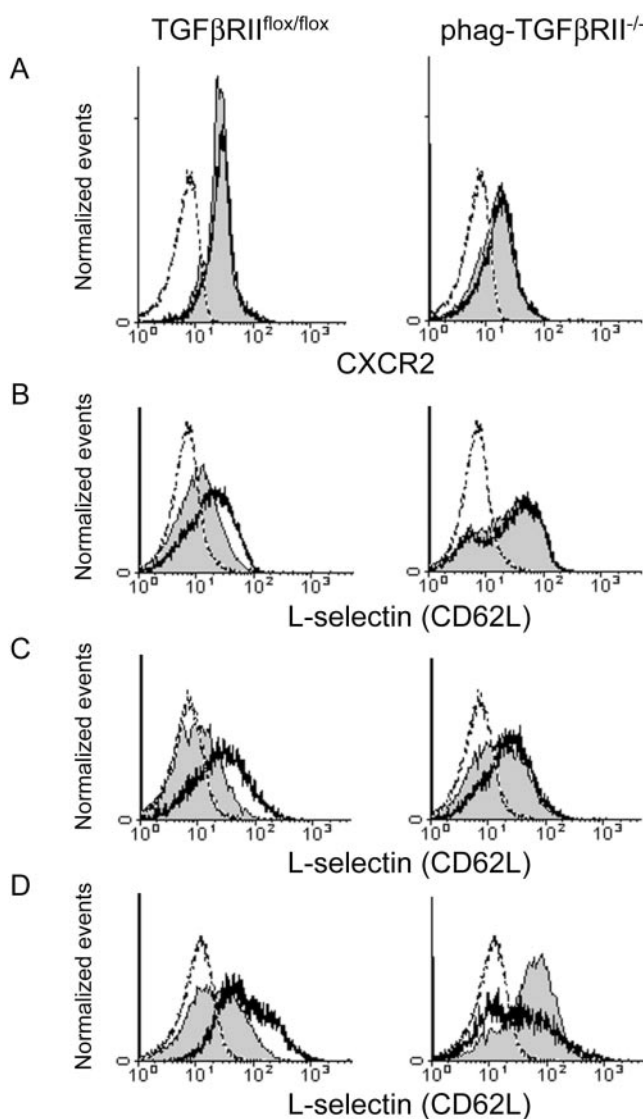

Fig. 6 Stimulation of thioglycollate-elicited PMN with TGFβ *in vivo* reduces their expression of L-selectin. TGFβRII$^{flox/flox}$ mice (left) and phag-TGFβRII$^{-/-}$ mice (right) were injected intraperitoneally with thioglycollate. Ten hours later mice were treated intraperitoneally with either TGFβ1 or PBS/BSA for an additional 10 h. (**A**) Expression of CXCR2 in Gr-1$^+$ CD11b$^+$ PMN. Histogram overlays show comparable levels of CXCR2 in TGFβRII$^{flox/flox}$ and phag-TGFβRII$^{-/-}$ mice. Isotype control PE-ratIgG2a (dotted line), thioglycollate and TGFβ (grey shaded area), thioglycollate and PBS/BSA (black line). (**B**) L-Selectin expression in Gr-1$^+$ CD11b$^+$ PMN. Histogram overlays depict a reduction of L-selectin expression in TGFβ-treated TGFβRII$^{flox/flox}$ mice but not in phag-TGFβRII$^{-/-}$ mice. Isotype control FITC-ratIgG2a (dotted line), thioglycollate and TGFβ (grey shaded area), thioglycollate and PBS/BSA (black line). The percentage of L-selectin$^+$ Gr-1$^+$ CD11b$^+$ PMN in the different groups is as follows: TGFβRII$^{flox/flox}$ mice treated with TGFβ: 10.4%; TGFβRII$^{flox/flox}$ mice treated with PBS/BSA: 31.2%. In phag-TGFβRII$^{-/-}$ mice the respective values were 49.1 and 52.6%. (**C**) Gr-1$^+$ CD11b$^+$ PMN of animals injected intraperitoneally with tuberculin PPD show a decrease in L-selectin expression after TGFβ treatment in TGFβRII$^{flox/flox}$ mice compared with phag-TGFβRII$^{-/-}$ mice. Isotype control bt-ratIgG2a/SAV-APC-Cy7 (dotted line), tuberculin PPD and TGFβ (grey shaded area), tuberculin PPD and PBS/BSA (black line). In TGFβRII$^{flox/flox}$ mice treated with TGFβ: 5.1% of Gr-1$^+$ CD11b$^+$ PMN were L-selectin$^+$; in TGFβRII$^{flox/flox}$ mice treated with PBS/BSA: 34.6%, respectively. In phag-TGFβRII$^{-/-}$ mice the respective values were 32.2 and 35.3%. (**D**) Intraperitoneal injection of inactivated *S. pneumoniae* results in a decrease of L-selectin expression in Gr-1$^+$ CD11b$^+$ PMN in TGFβRII$^{flox/flox}$ mice treated with TGFβ (grey shaded area) compared with PBS-/BSA-treated TGFβRII$^{flox/flox}$ mice (black line). The percentage of L-selectin$^+$ Gr-1$^+$ CD11b$^+$ PMN is as follows: TGFβRII$^{flox/flox}$ mice treated with TGFβ: 31.7%; TGFβRII$^{flox/flox}$ mice treated with PBS/BSA: 72.0%. In phag-TGFβRII$^{-/-}$ mice the respective values were 67.6 and 40.0%.

TGFβRII on phagocytes may possibly lead to L-selectin-independent functional changes that improve both the recruitment of phagocytes into the CNS and bacterial clearance. This point needs to be clarified in future studies that should include antibody-mediated neutralization of TGFβ in *S. pneumoniae*-infected wild-type mice as well as studies on the effect of TGFβ on PMN *in vitro*.

The data presented point to the importance of TGFβ for promoting cerebrovascular complications in bacterial meningitis by impairing clearance of *S. pneumoniae*. The comparison of phag-TGFβRII$^{-/-}$ mice with TGFβRII$^{flox/flox}$ mice shows that the risk of developing intracerebral vasculitis with haemorrhages—which lead to brain oedema and thereby cause increased ICP and cerebral hypoperfusion—increases with a significant load of *S. pneumoniae* in the CNS, but not with the presence of high numbers of neutrophils. In patients with bacterial meningitis cerebrovascular complications are frequent and seen in around 20% of patients (Kastenbauer and Pfister, 2003; Weisfelt *et al.*, 2006). In pneumococcal meningitis, adverse outcomes with ischaemic or haemorrhagic stroke are observed mainly in patients with low CSF PMN counts and high bacterial titres (Giampaolo *et al.*, 1981; Scheld *et al.*, 1982; Kastenbauer and Pfister, 2003; Weisfelt *et al.*, 2006). This constellation is promoted by TGFβ in TGFβRII$^{flox/flox}$ mice with *S. pneumoniae* meningitis, the pathway being blocked in phag-TGFβRII$^{-/-}$ mice.

The balance between pro- and anti-inflammatory mediators is critical both for preventing the innate immune response from becoming destructive to the host and for initiating repair mechanisms. TGFβ has been suggested to be one of the cytokines that counteracts the inflammatory response. TGFβ causes suppression of H_2O_2 release, production of inflammatory cytokines and expression of inducible nitric oxide synthase (*see* Introduction). In rats injected with *Salmonella typhosa* LPS, TGFβ1 arrested LPS-induced hypotension and mortality (Perrella *et al.*, 1996). It is remarkable that even when infecting phag-TGFβRII$^{-/-}$ mice with *S. pneumoniae,* which leads to increased recruitment of PMN to the CNS, the absence of TGFβ signalling on phagocytes does not represent a risk factor for excessive production of leucocyte-derived inflammatory mediators and associated multi-organ inflammation, disseminated intravascular coagulation and organ failure. Thus, TGFβ is not a key player in the immune homeostasis of the activated phagocyte.

In summary, our results suggest that TGFβ impairs the innate immune response by hindering the recruitment of phagocytes to sites of infection, which results in decreased clearance of infectious agents. Owing to this effect, TGFβ promotes inflammatory complications including cerebral vasculitis, brain oedema and increased ICP in bacterial meningitis. The data presented here raise the possibility that antibodies to TGFβ, TGFβ neutralizing molecules such as decorin or TGFβ receptor blockers may be valuable for the treatment of patients that have a high burden of bacteria but only low numbers of PMN in the CSF.

Acknowledgements

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