Both *MHC* and *non-MHC* genes regulate inflammation and T-cell response after traumatic brain injury

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ABSTRACT

Genetic regulation of autoimmune neuroinflammation is a well known phenomenon, but genetic influences on inflammation following traumatic nerve injuries have received little attention. In this study we examined the inflammatory response in a rat traumatic brain injury (TBI) model, with a particular focus on major histocompatibility class II (MHC II) presentation, in two inbred rat strains that have been extensively characterized in experimental autoimmune encephalomyelitis (EAE); DA and PVG. In addition, *MHC* and *Vra4* congenic strains on these backgrounds were studied to give information on *MHC* and *non-MHC* gene contribution. Thus, allelic differences in *Vra4*, harboring the *Ciita* gene, was found to regulate expression of the invariant chain at the mRNA level, with a much smaller effect exerted by the *MHC* locus itself. Notably, however, at the protein level the *MHC* congenic PVG-RT1^{av1} strain displayed much stronger MHCII$^+$ presentation, as shown both by immunolabeling and flow cytometry, than the PVG strain, dwarfing the effect of *Ciita*. The PVG-RT1^{av1} strain had significantly more T-cell influx than both DA and PVG, suggesting regulation both by *MHC* and *non-MHC* genes. Finally, in terms of outcome, the EAE susceptible DA strain displayed a significantly smaller resulting lesion volume than the resistant PVG-RT1^{av1} strain. These results provide additional support for a role of adaptive immune response after neurotrauma and demonstrate that outcome is significantly affected by host genetic factors.

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1. Introduction

Traumatic brain injury (TBI) is the leading cause of neurological disability in the world, especially among young persons. In TBI the primary injury is followed by a phase of secondary degeneration characterized by a number of cellular and molecular events that include ischemia, edema, glutamate excitotoxicity, free radical production, growth factor deprivation, apoptosis, reactive gliosis, accumulation of immune cells and production of various inflammatory mediators, all of which may create additional tissue damage beyond the zone of primary injury. The inflammatory response in TBI is biphasic, with a first phase directly after the injury with extravasation of neutrophils and blood–brain barrier damage and a subsequent phase a few days later that can persist for months after the injury, characterized by the activation of resident cells such as astrocytes and microglia, transmigration of leukocytes and blood-derived phagocytes into the brain parenchyma and production of cytokines and chemokines (Lenzlinger et al., 2001; Morganti-Kossmann et al., 2007). Studies with anti-

inflammatory agents suggest that the innate immune response can be both detrimental and beneficial for the outcome and that the timing of therapy in relation to the dynamics of inflammatory events is crucial (Marklund et al., 2006). Both major histocompatibility (MHC) class I and II antigens are upregulated following experimental and human TBI, with MHC class II antigens being present mainly on microglia (Holmin et al., 1995, 1997; Oehmichen et al., 2009). Together with additional qualities of the CNS microenvironment, in particular degree of microglia maturation and the presence of co-stimulation, this may lead to a subsequent T-cell response (Aloisi et al., 2000, 1998; Becher et al., 2000). T cells are recruited to the CNS after experimental brain trauma and myelin autoreactive T-cell responses have been documented after TBI in the human (Clausen et al., 2007; Cox et al., 2006). Whether the injury-induced adaptive immune response after mechanical nerve injuries is innocuous, detrimental or even beneficial has been disputed (Popovich et al., 1996; Schwartz et al., 2009). However, in a landmark study the group of Schwartz could demonstrate that transfer of myelin reactive T cells substantially increased the number of surviving retinal ganglion cells after optic nerve crush (Moalem et al., 1999). Protective effects of adaptive immunity has since then been reproduced in many other experimental models and contexts, see e.g. (Hammarberg et al.,

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2000; Hauben et al., 2000; Kipnis et al., 2003). However, in most cases the studied models required active immunization protocols or transfer of encephalitogenic cells and it is still unclear to what degree this occurs in unprimed settings.

Strain-dependent differences in susceptibility to experimental autoimmune encephalomyelitis (EAE) have been recognized for many years and most of this effect can be linked to different variants of the MHC (in the rat referred to as RT1), in particular the class II region coding for MHC class II molecules (Weissert et al., 1998). For example, the DA (RT1^{av1}) strain is susceptible in almost all EAE induction protocols regardless of CNS autoantigen, whereas the PVG strain (RT1^c) strain is resistant. If qualitative aspects of the autoimmune response are categorically dependent on the ability of the MHC haplotype to present a certain antigen, non-MHC genes will exert an additional influence on the magnitude of this response (Weissert et al., 1998). It has not been known if these genetic aspects also play a role for outcome after mechanical nerve injuries. In our prior studies we did not detect any significant MHC dependent genetic influences in a F2 (DAxPVG) intercross on outcome measures that included expression of class II molecules (Lidman et al., 2003). However, this was studied in a ventral root avulsion model where immune cell infiltration is very limited (Lidman et al., 2003; Piehl et al., 1999). More recently, we compared the outcome of a spinal cord contusion injury in DA and PVG rats demonstrating a significantly better motor function in the EAE susceptible DA strain (Birdsall Abrams et al., 2007). The inflammatory response was not characterized in detail, but there were no discernible differences in motor function in animals differing only in MHC. Interestingly, however, we recently described a prominent MHC dependent genetic effect on neuropathic pain following nerve injury in the same two strains, suggesting that MHC regulated responses may operate in the absence of actively induced CNS autoantigen directed inflammation (Dominguez et al., 2008).

The aim of the present study was to perform a more detailed characterization of the effect of naturally occurring genetic differences on the inflammatory response in experimental TBI. We describe a conspicuous MHC haplotype dependent effect on MHC class II presentation, both non-MHC and MHC dependent effects on the T-cell influx and mainly non-MHC regulation of the resulting tissue injury, demonstrating that genetic factors are of relevance also for determining outcome after traumatic nerve injuries.

2. Methods

2.1. Animals

The DA (RT1^{av1}) strain was originally provided by Professor Hans Hedrich (Medizinische Hochschule, Hannover, Germany), while PVG (RT1^c) and the PVG-RT1^{av1} strains were obtained from Harlan UK Ltd (Blackthorn, UK). The DA.PVG-Vra4 and PVG.DA-Vra4 strains were obtained from a DAxPVG-RT1^{av1} advanced intercross line as previously described (Harnesk et al., 2008). Repeated backcrossing to the respective recipient strain was performed to create congenics carrying theoretically <0.1% of the donor genome outside the VRA4 locus. The animals were bred in a barrier animal facility under pathogen-free and climate-controlled conditions with 12 h light/dark cycles, housed in polystyrene cages with wood shavings and fed with standard rodent chow and water ad libitum. All experiments in the study were approved by the local ethical committee for animal experimentation (Stockholms Norra Djurförsöksetiska Nämnd).

2.2. Surgery

A total of 150 male rats weighing approximately 230–300 g, at an age of 8–12 weeks, were anesthetized by intraperitoneal injection of 2.7 ml/kg of a mixture of Hypnorm (fluanisone, 10 mg/ml, fentanyl citrate, 0.315 mg/ml; Janssen, Oxford, UK), Dormicum (midazolam, 1 mg/ml, Roche) and water. In addition, 0.2 ml of Marcain (bupivacaine, 5 mg/ml, AstraZeneca, Södertälje, Sweden) was injected subcutaneously in the sagittal midline of the skull before the skin incision was made. The rats were placed in a stereotactic frame and a 2 mm craniotomy was drilled 3 mm posterior and 2.3 mm lateral to the bregma. A standardized parietal contusion was made by letting a 24 gram weight fall onto a rod with a flat end diameter of 1.8 mm from a height of 7 cm in contused rats, while surgery in sham rats was finished after drilling (Feeney et al., 1981). In contused rats, the rod was allowed to compress the tissue a maximum of 3 mm. All animals were sacrificed with CO_2 and perfused with cold PBS containing Heparin (LEO Pharma AB, Malmö, Sweden) (10 IE/ml). Rats were sacrificed at 1 day, 6 days and 18 days after the injury.

2.3. Quantitative real-time PCR (qRT-PCR)

We performed weight drop lesion to 5 to 8 animals in each group and 4 animals in each group were sham operated. Quantitative RT-PCR was performed 6 days after the operation. A sample corer (Fine Science Tools, Heidelberg, Germany) was used to collect a $5 \times 5 \times 6$ mm piece of tissue consisting of the contusion core, the pericontusional cortex, the underlying hippocampus and part of the thalamus. The collected tissue was dissected in two parts, lysed in Matrix D tubes (MP Biomedicals, Irvine, CA) on a FastPrep homogenizer (MP Biomedicals) and resuspended in RLT buffer (Qiagen, Hilden, Germany) for total RNA preparation. Total RNA was extracted using a RNeasy Mini kit (Qiagen) and RNase-Free DNase Set (Qiagen), according to the manufacturer's protocols. Before cDNA preparation the two mRNA samples from the same rat were pooled together. cDNA was prepared by using reverse transcription with random hexamer primers (0.1 µg/ml; Gibco BRL, Invitrogen, Stockholm, Sweden) and Superscript Reverse Transcriptase (200U; Gibco). All RT-PCR runs were conducted using Bio-Rad SYBR® green (Bio-Rad) according to the manufacturer's instructions. Amplification was performed on a BIO-RAD iQ5 with a three-step PCR protocol. Primers were designed using the Primer Express (Perkin Elmer) or ABI Beacon software (Applied Biosystems). Primer specificity was assessed by using amplicon dissociation curves in each sample and the amplicon size was determined using gel electrophoresis combined with silver staining. Relative amounts of mRNA expression were calculated using a standard curve and the method for normalized expression provided in the iQ5 optical system software. The following targets were analyzed by RT-PCR; Cd74 (invariant chain, a MHC class II associated transcript), RT1-Ba, CD11b (C3 receptor), Mrf-1, Tnf and Cd3e. Gapdh and Hprt were used as housekeeping genes. Sequences of the used primers are shown in Table 1.

2.4. Immunohistology

A weight drop injury was performed in 4–6 animals in each strain studied at 1, 6 and 18 days after the injury. The brains were quickly dissected, frozen in isopentane containing dry ice and stored at −70 °C. Sections (14 µm) were serially cut in a cryostat at a distance of 1800 µm around the epicenter of the lesion and kept at −20 °C until further processing. After thawing at room temperature, the sections were fixed in acetone at −20 °C or 4% formaldehyde at room temperature. Endogenous peroxidase was quenched for 30 min in 0.3% hydrogen peroxide diluted in PBS. After washing with PBS (PBS was used in all washing steps), the sections were incubated at room temperature for 1 h with PBS containing 1% bovine serum albumin, 0.3% Triton X-100 and 0.1%