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PD-L1 (B7-H1) regulation in zones of axonal degeneration

Michael Lipp [a,b], Christine Brandt [a], Faramarz Dehghani [c], Erik Kwidzinski [a], Ingo Bechmann [b,*]

[a] *Institute of Cell Biology and Neurobiology, Department Exp. Neuroimmunology, Charité, 10098 Berlin, Germany*
[b] *Dr. Senckenbergische Anatomie, Institute I, Johann Wolfgang Goethe-University, Frankfurt/Main, Germany*
[c] *Dr. Senckenbergische Anatomie, Institute II, Johann Wolfgang Goethe-University, Frankfurt/Main, Germany*

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Abstract

Fibre tract injury evokes recruitment of antigen-presenting- and T cells, but does not cause autoimmune demyelination. This implies that immune tolerance to myelin is actively maintained or readily re-established. Using entorhinal cortex lesion (ECL) to induce axonal degeneration in the hippocampus of adult mice, we studied the induction of B7-H1 (PD-L1) in zones of axonal degeneration. This member of the B7-family has been shown to be expressed on parenchymal cells of various organs, where it strongly down-modulates the activity of T cells. Real-time reverse transcriptase (RT)-PCR revealed low mRNA levels in brain compared to lung and spleen under normal conditions. After ECL, a twofold increase could be observed. Immunocytochemistry revealed astrocytes as source of B7-H1, while immune positive microglia were not detected. Thus, axonal degeneration induces astrocytes to express B7-H1, a potent inhibitor of effector T cells.

Keywords: Axonal degeneration; Astrocytes; Immune tolerance; Immune privilege

Despite the presence of myelin-specific T lymphocytes in the normal repertoire [27] and their expansion following fiber tract injuries [23,10], none of the current experimental models of brain and spinal cord injury causes disseminated autoimmune neuroinflammation [14]. In man, metastatistical analysis revealed that multiple sclerosis (MS) is neither initiated nor exacerbated by brain trauma [8]. Conversely, a negative correlation between trauma and MS activation has been suggested [12]. Thus, the increasingly appreciated initiation of adaptive immune responses during various neuropathologies [24,18] is restricted to the area of degeneration and involves mechanisms eventually re-establishing immune tolerance [14].

Using entorhinal cortex lesion (ECL) to induce axonal degeneration in the hippocampus, we have previously demonstrated recruitment of T cells and haematogenous antigen-presenting cells into zones of axonal degeneration [4,1]. Nevertheless, immune tolerance is maintained even in mice with transgenic T cell receptors specific for myelin [14]. There is also drainage or transportation of axonal antigens into cervical lymph nodes, which coincides with apoptosis in this compartment [20]. Activated T cells may also be eliminated from the neuropil by astrocytes which express functional CD95L in zones of axonal degeneration [2,3,5]. Thus, axonal injury evokes a variety of mechanisms of immune tolerance [11] the net-effect of which can diminish the immune system's post-lesional capability to mount an immune response to myelin [20].

A recently detected mechanism of peripheral immune tolerance is the induction of the programmed death ligand 1 (PD-L1 or B7-H1). This cell surface glycoprotein belongs to the Ig superfamily. Its expression has originally been shown in the heart, skeletal muscle, placenta, and lung [7], but brain cells were also found to transcribe this gene [26,17]. Mice deficient in PD-1 develop lupus-like autoimmune diseases in organs, where its ligand is present under normal conditions suggesting its crucial role for the maintenance of immune tolerance [21]. In fact, PD-L1/PD-1 signalling was found to be critical in type I diabetes, feto-maternal tolerance and tumor-tolerance [22,25]. In the brain, blockading studies in the course of experimental allergic encephalomyelitis (EAE) also revealed a central role for PD-L1/PD-1 signalling in the termination of neuroinflammation [15,26,17,30,6]. Here, we tested whether PD-L1 is also

* Corresponding author at: Dr. Senckenbergische Anatomie, Institute for Clinical Neuroanatomy, Theodor-Stern-Kai 7, 60 590 Frankfurt/Main, Germany. Tel.: +49 30 450 528 267; fax: +49 30 450 528 902.
E-mail address: Bechmann@med.uni-frankfurt.de (I. Bechmann).

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induced in the course of axonal degeneration, which is characterized by a self-limiting, local autoimmune response to myelin [9,10].

Female, 8–12-week-old C57/bl6-J mice were purchased from Charles River (Charles River Laboratories, Inc., Wilmington, MA, USA) and housed under standard conditions with free access to food and water. Care was taken to minimize any pain and discomfort to animals. Experiments were approved by the state of Berlin.

For ECL, C57/bl6-J mice were deeply anesthetized using a Ketamin cocktail (20% Ketamin and 8% Rompun in 0.9% NaCl; doses 0.01 ml/g). The left medial entorhinal cortex was lesioned using a 2 mm broad stainless steel blade. The medial edge of the knife was adjusted to the following coordinates as measured from λ: anterior–posterior: 0.5 mm; lateral: 2.0 mm; dorsoventral: down to the base of the skull. EAE tissue from animals in the acute phase of the CNS inflammation was provided from a previous study [13].

mRNA expression was studied on days 1, 2, 3, 5, 7 and 14 after ECL and in unlesioned controls. The anesthetized animals were perfused with ice cold 0.9% NaCl and the brains were frozen immediately in liquid nitrogen. For RNA extraction brain tissue was first ground in liquid nitrogen with a mortar and pestle. Total RNA extraction was performed using TRItidy G isolation reagent (AppliChem, Darmstadt, Germany; 0.01 ml/mg brain homogenate per vial) according to the manufacturer's manual. For first-strand cDNA synthesis, 2 μg of total RNA was dissolved in 18 μl of double-distilled water ($H_2O_{dd.}$). A total of 0.4 μl of random primers (500 ng/μl, Promega, Madison, WI, USA) was added to each sample before incubation at 75 °C for 10 min. Samples were cooled on ice and added to a mixture consisting of 5× First Strand Buffer (8 μl/sample; Invitrogen, Carlsbad, CA, USA), DTT (0.1 M, 4 μl/sample; Invitrogen) deoxyNTPs (dNTPs) (2.5 mM, 4 μl/sample; Promega), DNAase (2 U/μl, 2 μl/sample; Ambion, Austin, TX, USA) and RNAasin (40 U/μl, 0.5 μl/sample; Promega). The solution was incubated at 37 °C for 30 min, then heated up to 75 °C for 5 min for DNAase inactivation. Samples were cooled on ice and Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT, 200 U/μl, 1 μl/sample; Promega) and RNAase-inhibitor (40 U/μl, 1 μl/sample; Promega) was added. Samples were incubated at 42 °C for 60 min and finally heated up to 94 °C for 5 min.

For Real-time RT-PCR, measurement of gene expression was performed using the ABI prism 7000 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). Primers (TIB MOLBIOL Syntheselabor GmbH, Berlin, Germany) were designed to span exon–exon junctions to prevent amplification of genomic DNA. Amplicons are <150 bp to enhance efficiency of PCR amplification. Relative quantification of specific gene expression was performed by two-step real-time PCR using cDNA as a template. Templates were multiplied using Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA) by adding AmpErase Uracil N-glycosylase (Applied Biosystems) for inhibiting non-specific products and false positive results. Real-time PCR of cDNA specimens was conducted in a total volume of 25 μl with primers at optimized concentrations. Thermal cycler parameters were 2 min at 50 °C, 10 min at 95 °C, and 45 cycles of denaturation at 95 °C for 30 s followed by annealing/extension at 60 °C for 1 min. Samples were normalized to GAPDH as housekeeping gene to account for the variability in the initial concentration of the total RNA and conversion efficiency of the RT reaction [29]. Product specificity of the PCR products and quality of primers were confirmed by agarose gel electrophoresis and dissociation curve analysis. All PCR assays were performed in triplicate. Standard curves were generated for PD-L1 and GAPDH and were found to have a PCR amplification efficiency >90% as determined by the slope of the standard curves. The result for the relative gene expression was calculated using the $2^{-\Delta\Delta}$CT method [16]. For statistical analysis student's *t*-test was used, setting $p < 0.05$ as significant. Sequences of oligonucleotides used in this study are as follows:

PD-L1-for 5′-GAGCTGATCATCCCAGAACTGC
PD-L1-rev 5′-GACCGTGGACACTACAATGAGGA
GAPDH-for 5′-GCAACTCCCACTCTTCCACCTTC
GAPDH-rev 5′-CCTCTCTTGCTCAGTGTCCTTGCT

For immuncytochemistry animals were sacrificed at 1, 3, 5, and 7 days ($n = 3$) post lesion. Transcardial perfusion was performed with 50 ml NaCl followed by a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). Unlesioned mice ($n = 3$) and the contralateral hemisphere of lesioned animals served as controls. Brains were removed and postfixed overnight in the same fixative. Forty-micrometer sections were cut on a vibratome. Endogenous peroxidase was blocked using 3% H_2O_2 in phosphate buffer (PB) for 5 min and slices were washed three times in PB, and unspecific binding was blocked using 10% normal horse serum (NHS) and 0.5% Triton in PB. After incubation in this solution for 10 min, sections were washed again three times in PB and incubated at +4 °C overnight with a goat anti-mouse PD-L1 antiserum (R&D Systems, Minneapolis, MN, USA; 1:100) dissolved in 1% NHS and 0.5% Triton in PB. After washing three times in PB, sections were then incubated with a biotinylated, anti-goat secondary antibody (Vector Laboratories, Burlingame, CA, USA; 1:250) dissolved in 1% NHS and 0.5% Triton in PB for 90 min at room temperature. Binding of the primary antibody was visualized using an avidin–biotin-complex (ABC)–DAB method. Sections were dehydrated, embedded in Entellan (VWR International GmbH, Wien, Austria), and coverslipped. For co-localization, GFAP was labelled using DAB and PD-L1 was stained using Histogreen (Vector Labs) according to the instructions.

For quantification of the time course of PD-L1 transcription, real-time RT-PCR was performed. In accordance with others [17], there was a massive up-regulation in brain homogenate of animals in the acute phase of EAE (Fig. 1A). In comparison, the elevation in lesioned hemispheres studied on days 1, 2, 3, 5, 7 and 14 after ECL was modest (Fig. 1B). A significant increase was measured on day 7 ($p < 0.05$, Student's *t*-test). At this time, however, the levels were still much lower than those detected in normal spleen and lung (Fig. 1C). By day 14, levels had returned to control levels (Fig. 1B). One has to keep in mind, however, that the complete hippocampus was used for