Detrimental Contribution of the Immuno-Inhibitor B7-H1 to Rabies Virus Encephalitis

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Rabies virus is the etiological agent of an acute encephalitis, which in absence of post exposure treatment is fatal in almost all cases. Virus lethality rests on its ability to evade the immune response. In this study, we analyzed the role of the immuno-inhibitory molecule B7-H1 in this virus strategy. We showed that in the brain and spinal cord of mice, rabies virus infection resulted in significant up-regulation of B7-H1 expression, which is specifically expressed in infected neurons. Correlatively, clinical rabies in B7-H1−/− mice is markedly less severe than in wild-type mice. B7-H1−/− mice display resistance to rabies. Virus invasion is reduced and the level of migratory CD8 T cells increases into the nervous system, while CD4/CD8 ratio remains unchanged in the periphery. In vivo, neuronal B7-H1 expression is critically depending on TLR3 signaling and IFN-β because TLR3−/− mice—in which IFN-β production is reduced—showed only a limited increase of B7-H1 transcripts after infection. These data provide evidence that neurons can express the B7-H1 molecule after viral stress or exposure to a particular cytokine environment. They show that the B7-H1/PD-1 pathway can be exploited locally and in an organ specific manner—here the nervous system—by a neurotropic virus to promote successful host invasion. The Journal of Immunology, 2008, 180: 7506–7515.
Materials and Methods

Ab and reagents

Biotinylated anti-mouse B7-H1 (clone MIH5) and biotinylated anti-human B7-H1 (clone MIH2) were from eBioscience. Anti-human B7-H1 NH2 terminus (H-130) Ab was from Santa Cruz Biotechnology. MAP 2/ab specific Ab was purchased from Abcam. FITC-conjugated rabbit anti-RABV nucleocapsid Ab was from Bio-Rad. Alexa Fluor594-conjugated streptavidin was purchased from Molecular Probes. Cy3-conjugated donkey anti-rat IgG was from Jackson ImmunoResearch Laboratories. Fluoromount-G was obtained from Southern Biotechnology Associates, Fe-Block (anti Feγ III/II receptor rat mAb), anti-mouse CD3, CD19, CD8, and CD4 Abs were from BD Biosciences. Hot Start Taq polymerase and RNeasy Protect kit and Lipid Tissue Midi kit were purchased from Qiagen. SuperScript II RT was from Invitrogen. RNA Nano chips were obtained from Agilent. Recombinant human IFN-β was from Schering, rTNF-α from Pharmingen, and rIFN-γ from R&D Systems. Percoll was from Amersham Biosciences. OCT compound was from Miles.

Virus, human neural cells, and mice

The laboratory RABV strain CVS (ATCC vR995), a highly pathogenic RABV strain (27) causing fatal encephalomyelitis in mice, was propagated as described (28). HSV-1 strain KOS (29) was propagated on U373MG. B7-H1-/- and TLR3-/- mice were generated as described (30, 31). Six-week-old C57BL/6 from Janvier, B7-H1-/-, or TLR-3-/- female mice were inoculated i.m. in both hind legs, with 1 × 10^3 infectious particles of RABV. Disease progression was evaluated as described by scoring clinical signs and mortality (6). Mobility and mortality were scored as follows: 0 = normal control, 1 = ruffled fur, 2 = one paralyzed hind leg, 3 = two paralyzed hind legs, 4 = total paralysis (defined as the total loss of mobility), and 5 = death. Daily clinical score was obtained by adding individual scores. Dead mice were reported in the clinical score of the day of the death and thereafter (cumulative scores). Mobility and mortality were assessed by scoring clinical signs and mortality (6). Mobility and mortality were scored as follows: 0 = normal control, 1 = ruffled fur, 2 = one paralyzed hind leg, 3 = two paralyzed hind legs, 4 = total paralysis (defined as the total loss of mobility), and 5 = death. Daily clinical score was obtained by adding individual scores. Dead mice were reported in the clinical score of the day of the death and thereafter (cumulative scores). Disease progression was evaluated as described by scoring clinical signs and mortality (6). Mobility and mortality were scored as follows: 0 = normal control, 1 = ruffled fur, 2 = one paralyzed hind leg, 3 = two paralyzed hind legs, 4 = total paralysis (defined as the total loss of mobility), and 5 = death. Daily clinical score was obtained by adding individual scores. Dead mice were reported in the clinical score of the day of the death and thereafter (cumulative scores).

Results

Activation of B7-H1 mRNA and protein expression by RABV in human neurons

Previous transcriptome analysis of RABV-infected human neurons NT2-N has allowed us to identify the coinhibitor B7-H1 as a putative target of RABV to escape the host immune response. Further analysis of the neuronal expression of B7-H1 upon RABV infection was pursued in cell cultures of human NT2-N and in a human neuroblastoma cell line (SK-N-SH). NT2-N cell cultures are nearly pure populations of terminally differentiated postmitotic cells. They display biochemical, morphological, and functional characteristics of human neurons (40, 41). Control experiments were performed on the same cells infected with HSV-1. Kinetics of B7-H1 transcription in NT2-N after 1, 6, and 24 h post RABV infection were followed by real time PCR and compared with noninfected conditions (Fig. 1A). The yield of B7-H1 mRNA transcription

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Immunohistochemistry on hippocampus cultures was performed on 7-day cultured cells fixed in 10% paraformaldehyde and blocked with 10% normal goat serum, and 0.2% Triton X-100. Slides were incubated 1 h at RT with rat anti-mouse B7-H1 and anti-MAP2 mouse Ab then with Cy3-conjugated donkey anti-rat IgG and Cy2-conjugated donkey antimouse finally with 4′,6-diamidino-2-phenylindole. Coverslips were mounted in Fluoromount-G and the samples observed under a Leica DM500B UV. Images were processed with the Leica FW 4000 software.
B7-H1 molecules could also be detected by cytofluorimetry on the surface of RABV-infected SK-N-SH neuroblastoma after 24 h of infection (Fig. 1B). This evidence was obtained with two distinct B7-H1 Ab from two different sources. This means B7-H1 molecules have reached a location where they could potentially interact with their receptors. As a control experiment, NT2-N cells were infected with HSV-1 under the same experimental protocol as above. Despite a strong infection, as evidenced by RT-PCR (Fig. 1A, right panel) and immunocytochemistry (data not shown), no significant increase of B7-H1 mRNAs could be observed (Fig. 1A; HSV-1 24h). B7-H1 up-regulation in human neurons seems, therefore, specific of RABV infection.

**FIGURE 1.** B7-H1 is expressed in human neural cells upon RABV infection or after IFN-β or -γ treatment but not in cell lacking IFN-β. NT2-N, NT2-N/A, or SKN-SH were infected with RABV or treated with zIFN-β, -γ, or TNF-α. B7-H1, B7-DC, CD80, CD86, IFN-β expression and RABV infection were monitored by real time PCR. A. Kinetic of RABV infection (gray) and B7-H1 transcripts (black) in NT2-N. B7-H1 transcripts in 24 h HSV-1-infected NT2-N. Right panel. Infection was monitored by RT PCR in RABV- and HSV-1-infected cells by detecting RABV N protein or UL54 for HSV-1. 18S was used as a house-keeping gene. B. Surface expression of B7-H1 by cytofluorimetry in 24 h RABV-infected SK-N-SH (bold) compared with noninfected (solid) and irrelevant staining (dashed). Specific index of fluorescence was of 3. More than 88% of the SK-N-SH were infected. C. Relative gene expression of B7-H1, B7-DC, CD80, and CD86 were compared by real time PCR in noninfected NT2-N cells (white) and in 24-h RABV (dark) or HSV-1 (gray) infected NT2-N. 18S was used as a reporter gene. CD200R was used as a negative control (7). D. Effect of a 24-h treatment of zTNF-α (1.25 ng), 500 U of zIFN-γ, and IFN-β (1000 IU) on the B7-H1 (black) and B7-DC (gray) transcription by noninfected NT2-N/A. E. Viral load (N protein transcripts), B7-H1, IFN-β, and -γ transcription were compared in SK-N-SH and in SK-N-SH-CP, a variant of SK-N-SH naturally disabled for IFNs, were measured by real time PCR 2 days after infection. Data are presented as relative fold increase compared with noninfected conditions.

**FIGURE 2.** Hippocampal cultures express B7-H1 after incubation with IFN-γ and IFN-β. For 5–7 days, mouse fetal hippocampal cultures were either nontreated (NT) or treated with 500 U/ml IFN-γ or with 1000 U/ml IFN-β for 48 h. B7-H1 (red) and neurofilament MAP-2 (green) expressions were detected by immunocytochemistry. Nuclei are stained blue (4′,6-diamidino-2-phenylindole). Bar represents 100 μm.
Regulation of other B7-ligands after RABV infection in human neuronal cultures and in mixed astroglial/neuronal cultures

The neuronal expression of other members of the B7-family could be affected by RABV infection. We compared the expression level of B7-DC (PD-L2), B7.1 (CD80), and B7.2 (CD86) mRNA after RABV infection in NT2-N and in noninfected cells (Fig. 1C). RABV led to a modest increase of B7-DC (2-fold; black histogram) compared with noninfected cells (white histogram), whereas CD80 and CD86 transcripts were virtually unmodified because the level of transcription was similar to those of CD200R used as negative controls.

In the absence of infection, IFN-β or -γ trigger B7-H1 expression in human and mouse neural cell cultures

During the course of infection of NT2-N cultures by RABV, expression of B7-H1 mRNA appeared to correlate well with the development of the viral load (compare the two histograms in Fig. 1A). IFN-β mRNA monitored under the same conditions of infection followed a different time course. IFN-β transcription presented a 16-fold increase as early as 1 h post infection and peaked at 6 h post infection (3 × 10^4 increase; data not shown). It clearly preceded the induction of B7-H1 mRNA synthesis. In contrast, HSV-1 infection did not trigger B7-H1 expression and did not induce IFN-β expression either (data not shown and Ref. 36).

FIGURE 3. Expression of B7-H1 gene and protein in the mouse NS in the course of RABV infection. A, Expression of B7-H1 (solid line) and RABV N (dotted line) transcripts were analyzed in noninfected (day 0) and day 5 and 10 RABV-infected spinal cord and brain by real time PCR. Results are given as mean ± SD of relative fold increase of transcripts detected in samples of two or three mice. In this experiment, a gene (Insulin Like Growth factor), whose expression was barely not modified by RABV infection, had the following relative fold increases in the spinal cord: x1 at day 0; x2 at day 5, and x1 at day 7; and in the brain: x1 at day 0 and 5, and x1.7 at day 7. B, Immunohistochemistry was performed in day-7 brain section. B7-H1 (red) was expressed by noninfected cells and by infected (green) neurons (arrows). A total of 70% of infected neurons were B7-H1 positive. Bars represent 10 μm. C, Orthogonal confocal microscopy analysis showed that B7-H1 accumulated in the cytoplasma of infected neurons.
IFN-β might, therefore, be required for the induction of B7-H1 expression. We, therefore, analyzed whether the addition of IFN-β to noninfected human NT2-N and NT2-N/A triggers B7-H1 expression. Addition of IFN-γ and TNF-α was tested in parallel. IFN-β treatment (24 h, 1000U) led to a significant up-regulation of B7-H1 transcription in NT2-N (appr. 50-fold; data not shown) and in NT2-N/A (150-fold; Fig. 1D, black histogram), therefore, indicating that exogenous IFN-β can trigger B7-H1 expression in human neurons and cocultures of neurons and astrocytes. Addition of IFN-γ also caused an 80-fold increase of the expression of B7-H1 transcripts in NT2-N/A, whereas TNF-α has a limited effect (Fig. 1D). Of note, B7-DC transcription was only triggered to very limited amounts by IFN-γ, -β, and TNF-α in human neurons/astrocytes cultures (Fig. 1D, gray histogram).

Notably, B7-H1 protein expression was also up-regulated in cultures of mouse hippocampus neurons exposed to IFN-γ or -β for 48 h (Fig. 2).

In the absence of IFN-β expression, RABV does not trigger B7-H1 expression in human neural cell cultures

To test whether in the absence of IFN-β, RABV can still up-regulate B7-H1 expression, we used a human neuroblastoma cell line naturally disabled in the IFN expression (SK-N-SH-CP). We observed in these cells that in absence of IFN-β signaling, RABV cannot up-regulate B7-H1 expression anymore (Fig. 1E), supporting strongly the role of IFN-β in the control of B7-H1.

Expression and regulation of B7-H1 in the mouse NS after RABV infection

First, we monitored the expression and regulation of B7-H1 during NS infection by RABV by assessing brain and spinal cord specimens from animals at an early (day 5) and a late stage of infection (day 7) using real time RT-PCR as well as immunohistochemistry. As expected (11), B7-H1 mRNAs were virtually undetectable in the noninfected NS. In contrast, B7-H1 mRNA expression progressively increased during the course of infection and correlated with the invasion of spinal cord and brain by the virus (Fig. 3A). To identify the cellular source of B7-H1, we performed double immuno-staining analysis detecting infected neurons (in green) and B7-H1 (in red) in brain specimens at days 5 and 7. As shown in Fig. 3, B (x and y-axis) and C (x and y-axis and orthogonal analyses), infected neurons can express B7-H1 (upper panel). This expression occurs primarily in the cellular bodies of RABV-infected neurons and not in the neurites (lower panel of Fig. 3B). The vast majority of infected neurons (70%) can express B7-H1. B7-H1 was also expressed by noninfected cells (data not shown), which correspond to endothelial cells, GFAP+ astrocytes, CD11b+ monocyte/macrophages, and migratory CD3+ T cells (11, 21). The B7-H1 positive CD3+ T cells appeared only at late stages of infection (day 7).

IFN-β rather than IFN-γ is a key cytokine contributing to B7-H1 up-regulation in RABV-infected NS

After having demonstrated the expression of B7-H1 after RABV infection in neuronal cells in vivo and in vitro, we next questioned which cytokines or cytokine-inducing pathways were involved in this in vivo up-regulation. Experiments in wild-type (WT) mice showed that IFN-β and -γ expression in the CNS of infected mice (Fig. 4A) was concomitant with an increase of B7-H1 expression (see Fig. 3A). To further analyze the role of IFN-β in the up-regulation of B7-H1 after RABV-infection, we investigated mice with impaired IFN-β production. As a model, we initially used mice lacking receptor for IFNα/β (IFN-Re/β−− mice). However, in these mice, rabies is fulminant. All mice were dead 4 days after

FIGURE 4. RABV infection triggers the expression of IFN-β and -γ in the mouse spinal cord and brain. A. Mice were injected with RABV in the hindlimbs and perfused. Spinal cord and brain were removed and subjected to transcriptional analysis. Transcription of IFN-β (black squares) and IFN-γ (white circles) were analyzed by real time PCR in noninfected (day 0) and RABV-infected spinal cord and brain at days 5 and 7 after infection. Results are given as mean of relative fold increase of transcripts detected in NS specimen of two or three mice with SD. B, TLR3−/− and C57BL6 (WT) mice were infected with RABV. Transcription of B7-H1, IFN-β, and IFN-γ were analyzed by real time PCR in the brains of RABV-infected TLR3−/− (black histograms) and WT mice (gray histograms) at days 5 and 7 post infection. A total of three mice of each group were analyzed for each time point. SD is indicated. *, Levels of transcription are significantly different in TLR3−/− and WT brains (student’s t test, p < 0.005). Three separate experiments were performed.
infection, a period too short to study B7-H1 up-regulation in the brain (data not shown). We turned to TLR3−/− mice (31) because we noticed that upon RABV infection, IFN-β expression was significantly decreased in TLR3−/− mice brains compared with WT mice. TLR3−/− and WT mice were infected with RABV and sacrificed 5 and 7 days after infection. Transcripts of IFN-β, -γ, and B7-H1 were quantified in the brain by real-time PCR (Fig. 4B). We found that B7-H1 up-regulation at day 7 was significantly decreased in TLR3−/− mice brains in comparison to WT (152-fold increase in WT and 56-fold increase in TLR3−/− brains). This was concomitant with a limited up-regulation of IFN-γ for were quantified in the brain by real-time PCR (Fig. 4). Significantly decreased in TLR3−/− mice brains compared with WT (516-fold increase in WT compared with 113-fold increase in TLR3−/− mice). In contrast, IFN-γ up-regulation was not reduced but showed a significant increase (7-fold increase compared with IFN-γ transcription in WT brains). However, despite the high expression of IFN-γ transcripts in TLR3−/− mice, B7-H1 expression was diminished. These observations support the hypothesis that IFN-β and not IFN-γ is a key contributor to the up-regulation of B7-H1 expression in the NS upon RABV infection.

**Contribution of B7-H1 to immune evasion in RABV infection**

We found that RABV specifically triggers the regulation and the surface expression of B7-H1 in infected neurons. Various reports have demonstrated that B7-H1 provides an inhibitory signal in the interaction with Ag-specific T cells. Importantly, B7-H1 is responsible for the exhaustion of antiviral T cell responses by triggering the anergy of activated T cells (19). We, therefore, tested whether this key molecule was involved in the immunoevasive strategy of RABV. To do so, we compared the progression and the outcome of RABV infection in mice lacking B7-H1 (30) with WT mice. B7-H1−/− mice and control WT C57BL6 mice (n = 8 in each group) were injected with a dose of virus expected to kill half of the mice (ED50). Mice were observed during 16 days post infection. Progression of the disease in the two groups of mice is illustrated by the cumulative clinical curves. Clinical signs (Fig. 5A) as well as mortality records (Fig. 5B) were assessed. In both groups, half of the mice started losing weight 6 days after infection (data not shown), indicating that they were infected. Consistent with our expectation for this injected dose, in both groups, half of the mice showed clinical signs of disease, including loss of hindlimbs mobility. However, in striking contrast to disease course observed in WT animals, clinical signs of encephalitis, such as hunchback, did not occur in sick B7-H1−/− mice. Mean cumulative clinical score was significantly decreased in B7-H1−/− mice compared with WT mice (Fig. 5A). By day 9, consistent with the dose of virus injected (dose 50), half of the WT mice died, whereas B7-H1 mice survived (Fig. 5B). Infection of mice with a dose that killed 100% of WT mice (dose 100) only killed half of the B7-H1 mice (Fig. 5B).

Neuroinvasiveness was compared in those mice, by measuring the amount of transcripts of viral N protein in the brain stem of RABV-infected WT and B7-H1−/− mice at days 5 and 10. A reference value of 1, taken for noninfected NS, was used for calibration. As shown in Fig. 5C, the viral transcription was drastically reduced in B7-H1−/− NS (38-fold less) as early as day 5 (83 value for N mRNAs in B7-H1−/− mice and 3.200 in WT). The difference was more pronounced at day 10 post infection, with 690-fold less viral transcription in the brain stem of B7-H1−/− mice than in WT (460-fold increase of N mRNAs for B7-H1−/− and 320,000-fold increase in WT mice). RABV neuroinvasiveness was, therefore, severely impaired in the absence of B7-H1.

RABV was also less severe in TLR3−/− mice than in WT mice (mortality was reduced by a 2-fold factor, 63% mortality in WT vs 34% mortality in TLR3−/− mice; data not shown). Of note, as shown in Fig. 4, B7-H1 was less transcribed in neural tissues of RABV-infected TLR3−/− mice than in WT mice.

**CD8+ T cell infiltration into the RABV-infected NS is maintained in the absence of B7-H1**

We previously observed that the severity of RABV infection was inversely correlated with the number of CD3+ and CD8+ T cells in the NS (6, 37). Conclusions of these previous studies were that highly neuroinvasive and pathogenic infections were associated with a severe drop in the number of CD3+ T cells of migratory cells into the NS, whereas high amounts of CD3+ or CD8+ migratory T cells were observed in poorly neuroinvasive and less pathogenic RABV infection.

The effect of RABV infection on the subpopulations of CD3+, CD4+, and CD8 in the pool of migratory T cells into the NS was further analyzed by cytofluorimetry and compared with those found in the periphery (spleen). WT and B7-H1−/− mice were infected with RABV. Splenocytes and NS immune cells were analyzed by cytofluorimetry 5 and 10 days postinfection in WT and B7-H1−/− mice. Analysis of CD4+ and CD8+ splenocytes indicated that RABV infection does not modify the CD4:CD8 ratio in the spleen during infection of WT and B7-H1−/− mice (Table II).

The nature of the cells invading the NS and the time course of the invasion were compared in the two groups of mice. Five days...
after RABV infection, and in contrast to what was observed in the absence of infection, mononuclear cells started to invade the infected NS (Fig. 6A). Invasion of CD3+ T cells was observed already at day 5. No difference was found between WT and B7-H1−/− mice (CD3+ T cells represented 55% of the migratory cells in WT and 62% in B7-H1−/− NS). As described previously (6), the pool of migratory CD3+ T cells in the WT NS dropped later (here at day 10 post infection; Fig. 6A). This decrease of CD3+ T cells was mainly due to a drastic disappearance of CD8+ T cells (CD8+ T cells represents only 3% of the CD3+ T cells migratory pool), whereas the pool of CD4+ T cells is more stable (Fig. 6, B and C). The drop of CD8+ T cells might account for the increase of the CD4:CD8 ratio, which rises from 2.9 at day 5 up to 5.8 at day 10 (Fig. 6D). In striking contrast to WT mice, neither the decrease of CD3+ T cell at day 10 nor the CD8+ T cell decrease were observed in infected B7-H1−/− mice (Fig. 6, A and B). At day 10, CD8+ T cells still account for 20% of CD3+ T cells in infected B7-H1−/− mice (compared with only 3% in RABV-infected WT mice; Fig. 6C). As a consequence, the CD4:CD8 ratio remained stable at day 10 post infection in the NS of B7-H1−/− mice (Fig. 6D). Apoptosis of migratory T cells in the brain of RABV-infected B7-H1−/− and WT mice was analyzed 5 days post infection by cytofluorimetry analysis taking reduction in size and increase of granulometry as a marker of T cell apoptosis as described (38) (Fig. 6E). Five days after infection, whereas 70% of migratory CD8+ T cells in WT mice were engaged in an apoptotic process, this percentage was reduced by 50% in the absence of B7-H1, indicating that, CD8+ T cells are partially protected against RABV-mediated apoptosis in the absence of B7-H1.

Therefore, the absence of B7-H1 correlated with the preservation of high amounts of CD3/CD8 migratory T cells and with a significant reduction of the viral invasiveness. These findings strongly suggest that up-regulation of B7-H1 taking place in infected neurons and in a WT context contributes to the exhaustion and eradication of CD8+ T cells and, thus, favors the escape of virus infection from the host immune response.

**FIGURE 6.** In the absence of B7-H1, the characteristic exhaustion of migratory CD3+ T cells into the NS of RABV-infected mice did not occur. Phenotyping of splenocytes or mononuclear cells infiltrating the NS of WT or B7-H1−/− mice were analyzed by cytofluorimetry on days 5 and 10 post infection. A, Kinetic of CD3+ T cell percentages among mononuclear cells invading the NS of B7-H1−/− (black) or WT mice (gray). B, Percentage at day 10 of CD4+ and CD8+ T cells among the migratory CD3+ T cells in the NS of B7-H1−/− (black) and WT (gray) mice. *p, different at p < 0.0005. C, Dot plot analysis of CD8+ T cells among migratory CD3+ T cells in the NS of B7-H1−/− and WT mice 10 days post infection. The 20% and 3% represent the % of CD8+ T cells in NS of B7-H1−/− and WT mice, respectively, whereas 72% and 37% represent the % of infiltrating CD3+ T cells in B7-H1−/− and WT mice, respectively. D, CD4:CD8 ratio among migratory cells into the NS of B7-H1−/− and WT mice. E, Apoptosis of migratory CD8+ T cells was decreased in the absence of B7-H1 expression (B7-H1−/− mice). Experiments were performed twice with two to three mice for each time point and condition.
Discussion

Our report identifies an important and new role of the coinhibitory B7-H1 pathway in antiviral NS immunity. Upon viral infection with RABV, B7-H1 expression was found to be up-regulated in NS cells, including neurons. Mice lacking a functional B7-H1 gene, B7-H1^{−/−} mice, displayed a marked resistance to rabies. Resistance to viral infection in B7-H1^{−/−} mice was associated with a reduced viral load in the NS and with an increase of infiltrating CD8$^+$ T cells into the NS, whereas immune response in the periphery was not different from WT mice.

RABV is a pathogen well-adapted to the NS, where it infects neurons. It is transmitted by the bite of an infected animal. It enters the NS via a motor neuron through the neuromuscular junction, or via a sensory nerve through nerve spindles. It then travels from one neuron to the next, along the spinal cord to the brain and the salivary glands. The virions are excreted in the saliva of the animal and can be transmitted to another host by bite (43). Thus, preservation of the neuronal network integrity is crucial for the virus. Successful invasion of the NS by RABV seems to be the result of a subversive strategy based on the survival of infected neurons (5, 44). This strategy includes protection against virus-mediated apoptosis and destruction of T and NK cells that invade the NS by redundant control of the expression of the immunosubversive molecules FasL and HLA-G (4, 6, 44). The present study strongly suggests that this strategy also includes CD8$^+$ T cell exhaustion/death by the B7-H1/PD-1 pathway. CNS-derived B7-H1 might be operative in inhibiting local T cell expansion or even directly “eliminate” T cells, e.g., by apoptosis (6) as demonstrated in other systems, such as cornea (20). Previous studies indicated that T cells entering the NS underwent apoptosis in the course of RABV infection in mice (5, 6). We observed that in WT mice, the CD8$^+$ T cells entering the NS encounter apoptosis (Fig. 6E); this number was reduced by 50% in the B7-H1^{−/−} mice, indicating that B7-H1 contributes to the apoptosis of effector T cells infiltrating the infected NS. B7-H1-mediated killing of CD8$^+$ T cells was obtained in cocultures of MHC and B7-H1 expressing neurons with activated OT-1 CD8$^+$ T cells (S. Meuth, O. Simon, and H. Wiendl, manuscript in preparation). The fact that reduction of CD8$^+$ T cell apoptosis was not completely abrogated in RABV-infected B7-H1^{−/−} mice may suggest that other proapoptotic factors were still expressed by B7-H1^{−/−}-infected neurons. The remaining CD8$^+$ T cells death could be the result of the expression of B7-DC the second receptor for PD-1, which expression was increased in neuronal cultures by RABV infection (Fig. 1D). Because B7-H1 is expressed by inflammatory cells (Ref. 11 and this manuscript), killing of CD8$^+$ T cells may also be a result of B7-H1-positive microglia and macrophages. Killing of CD8$^+$ T cells would, thus, be expected to occur in any NS infection in which IFN is produced. Microglia is activated, and macrophages infiltrate the NS. However, in such an infection model (West Nile infection), we did not observed any decrease in the number of migratory T cells (45). Thus, it is unlikely that B7-H1 expressed by inflammatory cells is a major pathway to promote migratory T cell death in the infected NS.

Our findings are in good accordance with recent observations demonstrating that absence of B7-H1 leads to a more efficient elimination of adenovirus in mice (46) and that in human rhinovirus infection, the B7-H1/PD-1 pathway triggers a hindered immune response in the respiratory tract (23). They are also reminiscent of what has been observed in Schistosoma where infected macrophages up-regulate B7-H1 molecules thereby exhausting the host immune response (47). Very recently, PD-1 on T cells has been attributed to an exhausted phenotype of Ag-specific CD8$^+$ T cells, a phenomenon relevant in chronic infections with immunoevasive viruses, such as chronic lymphocytic choriomeningitis virus, (19), HIV (48, 49), and hepatitis C virus (50, 51). But, our results also stress a specific and important aspect of RABV infection, its “organ-specificity”. In this study, the B7-H1/PD-1 pathway is specifically manipulated upon infection in the NS. Expression of B7-H1 is modulated in this organ as well as B7-H1-induced changes in CD4:CD8 ratio in the NS. This takes place despite the fact that no change in the CD4:CD8 ratio occurred in the periphery and despite the fact that WT mice as well as B7-H1^{−/−} mice develop similar RABV-specific serum Abs (data not shown), strongly suggesting that mounting of primary immune response against RABV was not altered by the absence of B7-H1. We can conclude that it is the specific B7-H1 overexpression in the RABV-infected NS that contributes to the immunoevasiveness of the virus. However, among neurotropic viruses, up-regulation of B7-H1 could not be shared by every virus because, as reported here, HSV-1 did not alter B7-H1 expression and only partially counteracts and escapes the host immune responses (7).

We observed that mice lacking TLR3 showed a reduced B7-H1 expression, suggesting that TLR3 signaling was involved in B7-H1 regulation. Because TLR3^{−/−} mice develop a less severe form of RABV it can be hypothesized that TLR3 signaling pathway facilitates the immunoevasion. If this is the case, it will be reminiscent of the finding that in tumor cells, TLR4 signaling enhances immune suppression in vitro and that converse blockade of TLR4 prolongs the survival of mice grafted with tumor (52). Involvement of TLR signaling in inducing B7-H1 expression has been observed in tumor cells where ligation of TLR2, 4, and 9 with agonists (peptidoglycan, LPS, and double strand decoy oligodeoxynucleotides, respectively) induced B7-H1 expression (52, 53). We recently established that human neurons express TLR3 and can mount a chemoattractive, inflammatory, and antiviral-including IFN-β responses after RABV infection. TLR3 expression was found to be enhanced by RABV infection in human neurons in cultures as well as in autopsied rabies cases (36, 54). Whether TLR3 signaling is also involved in B7-H1 deserves further investigation. Nevertheless, because signalization through TLRs, such as TLR3, leads to the mounting of an IFN response, IFN response may be one arm of the TLR3-mediated regulation of B7-H1.

Both type I and II IFNs (IFN-β and γ) have been described to up-regulate B7-H1 transcripts in human DCs and monocytes (12), IFN regulatory factor 1 (IRF1) is a crucial factor in the IFN-γ-induced up-regulation of B7-H1(55). Our results indicate that IFN-β and -γ also control B7-H1 expression in human neurons and astrocytes as well as in mouse hippocampal neuronal cultures. In the course of RABV infection, IFN-β production by the infected NS precedes the onset of B7-H1 transcription, which is virtually absent in noninfected NS. Severely impaired IFN-β production (as observed in RABV-infected TLR3^{−/−} mice) is associated with a decrease in B7-H1 transcription. Therefore, B7-H1 up-regulation in the NS occurs as a response to or at least requires the presence of a specific “inflammatory” or cytokine milieu. The fact that HSV-1 does not trigger B7-H1 expression in HSV-1-infected neurons is fully consistent with the contribution that IFN-β exerts in the regulation of B7-H1 transcription, because HSV-1 strictly inhibits host IFN response (36, 56). It is surprising that B7-H1 expression in TLR3^{−/−} brains was still reduced despite the production of large amounts of IFN-γ transcripts in these tissues. This may indicate that—at least in vivo—B7-H1 expression requires a production of IFN-β and -γ above a certain threshold and/or that synergetic effects between the two cytokines are required.

In any case, these observations suggest that B7-H1 up-regulation in the NS occurs as a response to or in the presence of a
specific “inflammatory” cytokine milieu (11). In the instance of autoimmune NS inflammation, expression of B7-H1 is localized within areas of strongest inflammation and is considered to act as a “negative” regulatory feedback loop for keeping the “anti-inflammatory milieu” in the NS (4). Such a tissue tolerance function of B7-H1 has similarly been postulated in other parenchyma-teous organs, as recently shown for the pancreas in a disease model of autoimmune diabetes (9, 10). Although the inhibitory function of tissue related B7-H1 is beneficial in the instance of autoim-mune inflammation aggression, it has opposite effects in the case of viral infection; here, the virus diverts a feed back loop to its own strategy. In the case of RABV, a pathogen well adapted to the NS, we propose that B7-H1 acts as a viral target for immune evasion.

Our data support a sequential scheme of events after NS infec-tion by RABV contributing to RABV immunoevasement. Early in NS infection by RABV, NS cells mount an innate immune response including TLR3 signaling and IFN-β production leading to B7-H1 expression. Infected neurons that express TLR3 can directly contribute to the IFN-β production. B7-H1 protein subsequently reaches the cell surface of the infected neurons, where it could interact with its main receptor, PD-1, known to be expressed by T as well as B cells. Interaction of B7-H1 with PD-1 would then trigger the exhaustion of CD8+ T cells (e.g., reducing cell expansion or promoting active elimination) and, thus, favor the viral invasion of the NS. This pathway appears of crucial importance to ensure the progression of the disease in the NS because mice eliminate much more efficiently the invading virus when it is abrogated.

Taken together, our data provide evidence for an important role of the B7-H1/PD-1 pathway in some neurotropic infections. The demonstration of neuronal expression of B7-H1 after a RABV infection and the fact that mice lacking B7-H1 have a significant survival advantage clearly advocate the notion that B7-H1 is critically involved in the strategies used by RABV to escape the host immune response. Interference affecting the B7-H1/PD-1 interactions might then be envisioned as a possible therapeutic approach to strengthen and expand actual post exposure treatment of human rabies.

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References


