CTLA4 blockade elicits paraneoplastic neurological disease in a mouse model

Lidia M. Yshii,1,2 Christina M. Gebauer,1,* Béatrice Pignolet,1,3* Emilie Mauré,1 Clémence Quériault,1 Mandy Pierau,4 Hiromitsu Saito,5 Noboru Suzuki,5 Monika Brunner-Weinzierl,4 Jan Bauer6 and Roland Liblau1

*These authors contributed equally to this work.

CTLA4 is an inhibitory regulator of immune responses. Therapeutic CTLA4 blockade enhances T cell responses against cancer and provides striking clinical results against advanced melanoma. However, this therapy is associated with immune-related adverse events. Paraneoplastic neurologic disorders are immune-mediated neurological diseases that develop in the setting of malignancy. The target onconeural antigens are expressed physiologically by neurons, and aberrantly by certain tumour cells. These tumour-associated antigens can be presented to T cells, generating an antigen-specific immune response that leads to autoimmunity within the nervous system. To investigate the risk to develop paraneoplastic neurologic disorder after CTLA4 blockade, we generated a mouse model of paraneoplastic neurologic disorder that expresses a neo-self antigen both in Purkinje neurons and in implanted breast tumour cells. Immune checkpoint therapy with anti-CTLA4 monoclonal antibody in this mouse model elicited antigen-specific T cell migration into the cerebellum, and significant neuroinflammation and paraneoplastic neurologic disorder developed only after anti-CTLA4 monoclonal antibody treatment. Moreover, our data strongly suggest that CD8+ T cells play a final effector role by killing the Purkinje neurons. Taken together, we recommend heightened caution when using CTLA4 blockade in patients with gynaecological cancers, or malignancies of neuroectodermal origin, such as small cell lung cancer, as such treatment may promote paraneoplastic neurologic disorders.

1 INSERM UMR U1043 - CNRS U5282, Université de Toulouse, UPS, Centre de Physiopathologie de Toulouse Purpan, Toulouse, 31300, France
2 Department of Pharmacology, Institute of Biomedical Sciences I, University of São Paulo, 05508-900, Brazil
3 Department of Clinical Neurosciences, Toulouse University Hospital, 31059, France
4 Department of Experimental Paediatrics, University Hospital, Otto-von-Guericke University Magdeburg, 39120, Germany
5 Department of Animal Genomics, Functional Genomics Institute, Mie University Life Science Research Center, 2-174 Edobashi, Tsu, Mie 514-8507, Japan
6 Department of Neuroimmunology, Center for Brain Research, Medical University of Vienna, A-1090, Austria

Correspondence to: Roland Liblau, MD, PhD, Centre de Physiopathologie de Toulouse, Purpan Hospital, Place du Docteur Baylac TSA 40031, 31059 Toulouse Cedex 9, France
E-mail: roland.liblau@inserm.fr

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Abbreviations: HA = haemagglutinin; mAb = monoclonal antibody
Introduction

Paraneoplastic neurological disorders are immune-mediated diseases that develop in the setting of malignancy and offer a unique prospect to analyse the interplay between tumour immunity and autoimmune disease (Albert and Darnell, 2004; Steinman, 2014). In paraneoplastic neurological disorders, the pathogenic adaptive immune response targets proteins, so-called onconeural antigens, normally expressed by neurons and aberrantly expressed by tumour cells (Furneaux et al., 1990). Highly specific anti-neuronal autoantibodies in the serum and CSF represent key diagnostic biomarkers. When these autoantibodies recognize cell surface antigens, they most likely contribute to pathogenicity (Tuzun et al., 2009; Graus et al., 2010). In contrast, when onconeural antigens are localized intracellularly, T cells provide a beneficial anti-tumoural response but are implicated in inducing the deleterious autoimmune neurological reaction (Pellkofer et al., 2004; Pignolet et al., 2013). Paraneoplastic cerebellar degeneration, characterized by the selective loss of Purkinje neurons in the cerebellum, is an illustrative example of paraneoplastic neurological disorder targeting intracellular neuronal antigens (Albert et al., 1998). Paraneoplastic cerebellar degeneration develops, in particular, in patients with gynaecologic carcinomas that express the Purkinje neuron-specific CDR2 protein, with small cell lung cancer that expresses the neuronal HuD protein, or with testicular cancer that expresses the Ma2 antigen (Mason et al., 1997; Albert et al., 1998; Dalmau et al., 2004).

Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) is expressed transiently following activation of conventional T cells, and constitutively by regulatory T cells (Tregs). This receptor has a crucial role in limiting T cell responses (Allison, 2015). Therefore, therapeutic blockade of CTLA4 with antibodies has been tested to enhance anti-tumour immunity, and proved to be efficient in murine CD4+ T cells were incubated with anti-CD25 (clone PC61) and anti-CD62L antibodies. Naive CD4+ and CD8+ T cells were purified by negative selection using magnetic beads. Naive Ha-specific CD4+ and CD8+ T cells were cultured as described (Aslakson and Miller, 1992). CD4+ T cells were depleted by incubation with anti-CD4 (clone 24G2) monoclonal antibody (mAb) for 10 days, and T cells were injected intravenously into the in vivo setting, we assessed the impact of anti-CTLA4 therapy on development of paraneoplastic neurological disorder.

Materials and methods

Mice

6.5-TCR mice, CL4-TCR mice, L7-Cre, Mog-HA, and the Rosa-Stop-HA (Rosa26^{rmm(HA)}{ conditional knockin) mice have been described elsewhere (Kirberg et al., 1994; Morgan et al., 1996; Saito et al., 2005; Saxena et al., 2008). L7-Cre mice were back-crossed at least six times on the BALB/c background. We obtained the L7-HA mice by crossing L7-Cre with Rosa-Stop-HA mice. Mice were kept in specific pathogen-free conditions within the UMS006 animal facility, Toulouse, France. All procedures involving animals were in accordance with the European Union guidelines following approval by the local ethics committee.

Reverse transcription polymerase chain reaction

To assess influenza virus haemagglutinin (HA) expression, total RNA was extracted with TRIzol (Life), followed by reverse transcription (SuperScript III, Invitrogen). The cDNA was used as template for quantitative PCR using SYBR Green I master mix (Roche) on a LightCycler 480 thermocycler (Roche). The following primers were used to amplify HA (forward 5'AAACTCTTCCGGTCTTTCCA 3', reverse 5'GATAAGGTAGGCTGGGTGC 3'), Ctlta4 (forward 5'GCTTCTAGATTA CCCCTTCTGC 3', reverse 5'C GG CATGTTTCTGATCA 3'), and Hprt (forward 5'TTGCTCGAG ATGCATGAAGGA 3', reverse 5'TGAGAGATCAT CCA CGAAATATT 3'). HA mRNA expression was normalized to Hprt mRNA.

Cell line and tumour implantation

The BALB/c mouse 4T1 breast cancer cell line and its 4T1-HA derivative that expresses HA (a gift from D. Klatzman) were cultured as described (Aslakson and Miller, 1992). 4T1 or 4T1-HA cells (10^5) were injected subcutaneously into the left flank of mice. Tumours were measured with a Vernier calliper daily for 20 days, and the tumour size (w × l) is expressed as mm^2.

Haemagglutinin-specific T cells

Naive HA-specific CD4+ and CD8+ T cells were purified from 6.5-TCR and CL4-TCR mice, respectively. Spleens and lymph nodes were collected and T cells were purified (Dynabeads Untouched Mouse CD4+ or CD8+ Cells kit; Invitrogen). CD4+ T cells were incubated with anti-CD25 (clone PC61) monoclonal antibody (mAb) for Treg depletion. Naive T cells were further purified based on expression of CD62L (Miltenyi Biotech). Naive CD4+ and CD8+ T cells (10^7 each), depleted of Tregs, were injected intravenously into the
mice. Mice were assessed daily for weight and neurological signs.

**Anti-CTLA4 and anti-CD8 treatment**

For *in vivo* antibody therapy, the hamster anti-mouse CTLA4 mAb (UC10-4F10-11) was produced by culturing hybridoma cell lines in RPMI 1640 supplemented with low IgG foetal bovine serum. Antibodies were purified from supernatants using protein G purification (GE Healthcare). Mice were treated through intraperitoneal injections of anti-CTLA4 mAb (100 µg/mouse) at Days −1, 0, 2, 4, 6, 8, 10, 12, 14 and 16. In one experiment, depleting anti-CD8 mAb (33.6.7) or control IgG (200 µg/mouse) were injected intraperitoneally at Days 7, 9, 11, 13, 15, 17 and 19.

**Motor behavioural tests**

Motor coordination was assessed with a Rotarod device (Bioseb) with acceleration from 4 to 40 rpm over a 600 s period. For training, mice were tested on seven consecutive days, thrice daily. During the investigation period (Day 0 to Day 20), the latency to fall was measured for each mouse twice daily, and the longest time was recorded. The 100% represents the Day 0 value. To test the spontaneous motor activity an automated actimeter (ActiTrack) was used. This apparatus consists of four 22.5 cm² surface areas with 16 surrounding infrared beams coupled to a control unit. The activity was recorded for 10 min every day, and total distance travelled (m) was recorded.

**Purification and activation of cerebellum-infiltrating mononuclear cells**

Brain-infiltrating mononuclear cell purification was described previously (Martin-Blondel et al., 2015). Briefly, 15 days after tumour implantation, mice were perfused with phosphate-buffered saline (PBS) and their cerebellum was dissected. Mononuclear cells were collected and used for flow cytometry staining or *in vitro* stimulation with peptides. For the latter, cerebellum-infiltrating mononuclear cells were stimulated *in vitro* for 16 h with HA512-520 or control H-2-K^{d}-binding peptide (Cw370-179) for CD8^{+} T cells, and HA110-119 or control H-2-1A^{d}-binding peptide (OVA323-339) for CD4^{+} T cells. The cells were incubated with GolgiPlug^{TM} (1:1000; BD) and GolgiStop^{TM} (1:1000; BD) for 16 h.

**Flow cytometry analysis**

Lymph node cells, splenocytes, and tumour-infiltrating cells were stained with anti-Thy1.2 (53-2.1, BD), anti-CD45 (30-F11, Biolegend), anti-CD8α (53-6.7, BD), anti-CD4 (RM4-5, BD), and anti-CD152 (UC10-4B9, eBioscience) mAbs. Cerebellum-infiltrating cells were stained with the following antibodies: anti-Thy1.2, anti-CD45, anti-B220 (RA3-6B2, BD), anti-CD138 (28-1-2, BD), anti-CD8α, anti-CD4, anti-CD11b (M170, eBioscience), anti-IFN-γ (XMG1.2, BD), anti-TNF-α (MP6-XT22, BD), and anti-IL-17A (TC11-18H10, BD). 4T1 and 4T1-HA tumour cells were stained with anti-CD80 (16-10A1, eBioscience) and anti-CD86 (GL1, BD) mAbs and in agreement with previous data (Ruocco et al., 2012), we found no detectable CD80 and CD86 expression. Data were recorded on an LSRII Fortessa (BD Bioscience) and analysed with the FlowJo software (Tree Star).

**Immunohistochemistry**

The light microscopical stainings were performed as described (Bien et al., 2012; Bauer and Lassmann, 2016). The primary antibodies used were: anti-Calbindin D28K (1:2000, Swant), anti-CD3 (1:200, SP7, Zytomed), anti-CD8α (1:100, 45M15, eBioscience), anti-1b-1 (1:1000, Wako Chemicals), anti-Granzyme B (1:25, Santa Cruz), anti-activated caspase 3 (1:3000, BD), anti-Mac3 (1:200, BD), anti-β2-microglobulin (1:1000, Santa Cruz), and anti-C9neo (a kind gift by Dr Paul Morgan, Cardiff, UK). Staining for immunoglobulin (Ig) was performed using biotinylated donkey-anti-mouse Ig (Jackson). Quantification of immunohistochemistry data was performed by manual counting of Purkinje cells per mm² of Purkinje cell layer. In brief, for each mouse, the number of Purkinje cells was determined from five equally sized cerebellar cortex regions (central lobules II, III, simple lobule, ansiform lobule, paramedian lobule) and normalized for the length of the Purkinje cell layer.

**Immunofluorescence**

The fluorescent stainings were performed as previously described (Bauer and Lassmann, 2016). The primary antibodies used were: rabbit anti-Calbindin D28K (1:2000, Swant), mouse anti-Calbindin D28K (1:2000, Sigma), mouse anti-HA37-38 hybridoma supernatant (1:10), rabbit anti-IP3R1 (1:500, ThermoFisher), mouse anti-H-2-K^{d} (1:500, ThermoFisher), anti-Granzyme B (1:25, Santa Cruz) and rat anti-CD8α (1:50). Images were analysed using ImageJ software (NIH).

**Statistical analysis**

Statistical analyses for two sets of data with normal distribution and for paired data were performed using the Student’s *t*-test. Comparisons between multiple groups were performed using the one-way ANOVA (post-test Tukey). For tumour size and weight loss, data were analysed using the two-way ANOVA (post-test Sidak). The analyses were performed on Prism 6 (GraphPad Software). Groups were considered statistically different at a *P*-value < 0.05. The data are represented as mean ± SEM (standard error of the mean).

**Results**

**Model of paraneoplastic neurological degeneration**

To model experimentally paraneoplastic cerebellar degeneration, we designed a mouse model expressing the influenza virus haemagglutinin (HA) specifically in Purkinje cells. We crossed the Rosa-Stop-HA mice with the L7-Cre mice, which express the Cre recombinase specifically in Purkinje cells. In the resulting L7-HA mice, the stop cassette is
excised due to L7-controlled Cre expression, leading to selective expression of HA in Purkinje cells (Fig. 1A). Transcription of HA was detected by reverse transcriptase polymerase chain reaction (RT-PCR) in the cerebellum but not in other organs of L7-HA mice, including the rest of the CNS. Cerebellum of MOG-HA mice, which express HA in oligodendrocytes, was used as a positive control (Saxena et al., 2008) (Fig. 1B). Furthermore, by immunohistofluorescence, we confirmed that HA protein expression was confined to Purkinje cells in L7-HA mice as shown by the co-staining for HA and calbindin (Fig. 1C). As expected, HA expression was not detected in littermate control mice (wild-type).

Figure 1  Generation and characterization of the L7-HA mice. (A) Scheme of the L7-HA double knock-in mice. Top: In the Rosa-Stop-HA mice, a LoxP-flanked Stop cassette followed by the haemagglutinin (HA) sequence was introduced in the Rosa26 locus. Middle: In the L7-Cre mice, the Cre sequence was inserted in the Purkinje cell-specific L7/pcp2 gene. Bottom: In L7-HA mice, resulting from the crossing of the Rosa-Stop-HA and L7-Cre mice, the Cre-mediated recombination within the Rosa26 locus permits transcription of HA. (B) HA mRNA expression assessed by quantitative RT-PCR in different organs of L7-HA mice (black bar) or cerebellum of MOG-HA mice (open bar). One experiment representative of three is shown. n.d. = not detected. (C) Expression of HA protein in Purkinje cells from L7-HA mice. Double immunostaining for HA (green) and calbindin (Calb, red). Top row: Littermate control mouse (WT), bottom row: L7-HA mouse. Nuclear staining (DAPI). Scale bar = 50 μm. (D–G) CTLA4 expression was assessed by flow cytometry on Day 5 in transferred HA-specific Thy1.2+ CD4+ (D) or Thy1.2+ CD8+ (E) T cells in the lymph nodes of Thy1.1 congenic mice bearing 4T1 or 4T1-HA tumour, and in tumour-infiltrating total CD4+ (F) and CD8+ (G) T cells.
To further model paraneoplastic neurological disorder, L7-HA or wild-type mice were challenged with the 4T1-HA breast cancer cells that express the HA antigen. Given the key role of T cells as effectors of tumour control and paraneoplastic neurological disorder pathogenesis (Albert and Darnell, 2004; Dalmau and Rosenfeld, 2008; Pignolet et al., 2013), on the day of tumour implantation, mice were transferred with HA-specific naive CD4+ and CD8+ (CD4/CD8) T cells. CD4/CD8 T cells were capable of partially controlling tumour growth as compared to mice injected with tumour only (Fig. 2A), but they elicited no patent neurological manifestations.

**Anti-CTLA4 antibody triggers weight loss and reduced locomotion in L7-HA mice**

We next addressed whether treatment with a mAb blocking CTLA4 would increase anti-tumour immunity since a high proportion of T cells activated by the HA-expressing tumour expressed CTLA4/CD152 (Fig. 1D–G). Anti-CTLA4 mAb therapy protected mice almost completely from tumour outgrowth, regardless of their expression of HA in the cerebellum (Fig. 2A). Strikingly, L7-HA mice bearing 4T1-HA tumour and treated with anti-CTLA4 mAb lost weight significantly, from Day 15 onwards (Fig. 2B; \( P < 0.0001 \)).

To investigate whether mice also developed neurological signs, we evaluated motor activity. L7-HA mice bearing 4T1-HA tumour and treated with anti-CTLA4 mAb displayed a significant reduction in forced motor activity on the Rotarod during the diseased period (Fig. 2C; \( P < 0.0001 \)). Using the actimeter test, we also revealed reduced spontaneous locomotion in 4T1-HA tumour-bearing L7-HA mice that received anti-CTLA4 mAb (Fig. 2D). Importantly, no such clinical manifestations developed in 4T1-HA tumour-bearing wild-type mice treated with anti-CTLA4 mAb or in 4T1-HA tumour-bearing L7-HA mice not treated with anti-CTLA4 mAb (Fig. 2B–D).
Collectively, these results show that clinical signs resembling paraneoplastic neurological disorder occur upon CTLA4 blockade, only when the antigen is expressed both in the CNS and tumour.

**Therapeutic CTLA4 blockage induces cerebellar inflammation and Purkinje cell loss in L7-HA mice**

To uncover the structural defects underlying the phenotype caused by anti-CTLA4 mAb therapy in L7-HA mice, we performed neuropathological analyses. L7-HA mice implanted with 4T1-HA tumour and treated with anti-CTLA4 mAb showed a massive infiltration of T cells in the cerebellum (Fig. 3C). In addition, clear local microglial activation was present in these mice (Fig. 3F). Other brain regions were spared from inflammation (data not shown). Neither T cell infiltration nor microglial activation was detected in the cerebellum of wild-type mice treated with anti-CTLA4 mAb (Fig. 3A and D), or L7-HA mice not treated with anti-CTLA4 mAb (Fig. 3B and E).

CTLA4 blockade also resulted in loss of Purkinje cell soma and dendritic arborization in L7-HA mice (Fig. 3I), but not in control mice (Fig. 3G and H), as detected by calbindin immunostaining. This Purkinje cell loss was further confirmed by IP3R1 immunoreactivity (Fig. 3J), another characteristic marker of Purkinje cells (Miyata et al., 1999). No detectable Ctda4 mRNA expression was detected in the cerebellum of L7-HA mice, whereas a strong signal was evidenced in the spleen (data not shown). We then quantified the number of Purkinje cells by counting Calbindin+ cell bodies in 20 to 40 mm of Purkinje cell layer. A very significant reduction in Purkinje cells was observed in L7-HA mice treated with anti-CTLA4 mAb, in comparison to the other groups (Fig. 3K). The magnitude of Purkinje cell loss was similar between Days 20 and 45, underlying the acute nature of the paraneoplastic autoimmune process (data not shown). Overall, in the 32 L7-HA mice treated with anti-CTLA4 mAb, the mean loss of Purkinje cells was 40%, with marked interindividual variability ranging from 0 to 81% (Fig. 3K). Six L7-HA mice treated with anti-CTLA4 mAb presented normal Purkinje cell density, including five without cerebellar microglia activation and T cell infiltration. Collectively, these data suggest that the penetrance of disease is not 100% in our model. Thus, in L7-HA mice treated with anti-CTLA4 mAb, paraneoplastic neurological disorder is the consequence of massive cerebellar inflammation associated with the destruction of onconeural antigen-expressing Purkinje neurons.

**Immune mechanisms of Purkinje cell death in L7-HA mice with paraneoplastic neurological disorder**

To gain insight into the immune cells responsible for Purkinje cell loss the cerebellum of L7-HA mice with paraneoplastic neurological disorder was further studied by flow cytometry. Contrasting with control mice, the cerebellum of L7-HA mice with paraneoplastic neurological disorder harboured CD45\textsuperscript{high} CD11b\textsuperscript{low} lymphocytes as well as CD45\textsuperscript{high} CD11b\textsuperscript{high} myeloid cells (Fig. 4A). Some B cells (13.2 ± 3.4% of blood-derived CD45\textsuperscript{high} cells) including a small number of CD138+ plasma cells (range 24 to 90 cells/cerebellum, \(n = 14\)) were present on Day 15 after disease induction. T cells were the dominant population with a mean of 2000 (range 3 to 9219, \(n = 12\)) CD4\textsuperscript{+} and 1300 (range 2 to 7405, \(n = 12\)) CD8\textsuperscript{+} T cells. We then assessed the antigen specificity of these cerebellum-infiltrating T cells. Few CD4\textsuperscript{+} T cells reacted *ex vivo* to HA peptide by production of either IL-17 or IFN-\(\gamma\) (Fig. 4B). By contrast, cerebellum-infiltrating CD8\textsuperscript{+} T cells exhibited a marked HA-specific production of IFN-\(\gamma\) and TNF-\(\alpha\) (Fig. 4C). These data indicate that HA-specific CD8\textsuperscript{+} T cells are enriched in the cerebellum of L7-HA mice with paraneoplastic neurological disorder.

To further dissect the interaction between CD8\textsuperscript{+} T cells and Purkinje cells, immunohistological investigations were conducted in L7-HA mice exhibiting marked ongoing loss of Purkinje cells and their neighbouring dendritic tree (Fig. 5A and B). Numerous CD8\textsuperscript{+} T cells were localized in close contact with Purkinje soma or along their dendrites (Fig. 5C and D). In some instances, intimate interactions between CD8\textsuperscript{+} T cells and Purkinje cells were evidenced, with indentation of the neuronal surface and polarization of the cytolytic granules towards the CD8\textsuperscript{+} T cell/Purkinje cell interface (Fig. 5E and F). Possibility of direct targeting of Purkinje cells by HA-specific cytotoxic CD8\textsuperscript{+} T cells was further strengthened by *in situ* detection of MHC class I expression in Purkinje neurons (Fig. 5G). In L7-HA mice with paraneoplastic neurological disorder, some Purkinje cells exhibited DNA fragmentation (Fig. 5H) and caspase 3 activation (Fig. 5I), indicating ongoing apoptosis. Neuronophagia of cells with Purkinje-like morphology by macrophages/microglia was also detected (Fig. 5J). We also assessed Ig deposition and complement activation in the cerebellum of these mice. Whereas Ig leaked into the cerebellum due to blood–brain barrier disruption (Supplementary Fig. 1A), there was neither Ig binding (Supplementary Fig. 1B) nor C\textsubscript{3}neo deposition (Supplementary Fig. 1C) on Purkinje cells. Therefore, our data suggest that the humoral immune response does not play a main role in this model. Together, these findings indicate that cerebellar inflammation is associated with MHC class I expression on Purkinje cells, physical contact with CD8\textsuperscript{+} T cells, which polarize their lytic granules towards the neurons, strongly suggesting that cytotoxic T cells deliver a lethal hit to Purkinje cells in an antigen-specific manner. To further explore the *in vivo* role of CD8\textsuperscript{+} T cells in our model, we treated mice with a depleting anti-CD8 mAb. Whereas mice treated or not with control IgG exhibited loss of Purkinje cells (mean loss of 36 and 44%, respectively), the five mice treated with the depleting anti-CD8 mAb did not (data not shown).
Figure 3 Treatment with anti-CTLA4 antibody induces marked cerebellar inflammation and Purkinje cell loss selectively in L7-HA mice. (A–I) Histological analysis 20 days after transfer of naive HA-specific CD4$^+$ and CD8$^+$ T cells in L7-HA mice (B, C, E, F, H and I) or littermate controls (A, D and G) implanted with 4T1-HA, with (A, C, D, F, G and I) or without (B, E and H) anti-CTLA4 mAb treatment. Representative staining in brown for CD3$^+$ cells (A–C; top row), microglia (Iba-1, D–F; middle row), and Purkinje cells (calbindin, G–I; bottom row); nuclear counterstaining: haematoxylin (blue). Arrows in (I) point to loss of Purkinje cells. Scale bars = 100 μm. (J) Representative images of immunofluorescence confocal microscopy using co-staining with anti-calbindin and anti-IP3R1 antibodies to label Purkinje cells with independent markers. Nuclear staining was performed with DAPI. Scale bar = 50 μm. (K) Quantitative evaluation of the density of Purkinje cells in 4T1-HA tumour-bearing wild-type (WT) and L7-HA mice, treated or not with anti-CTLA4 mAb. Results are expressed as mean ± SEM of 6–32 mice per group from six independent experiments analysed at either Day 20 or 45 (**P < 0.001).
Finally, we wondered whether analogous immunological events could contribute to Purkinje cell targeting in human paraneoplastic neurological disorder in which autoantibodies have no demonstrated pathogenic effect. Confirming a previous report (Aboul-Enein et al., 2008), close apposition between CD8+ T cells and remaining calbindin+ Purkinje cells was detected in patients with anti-Hu or anti-Ma2 antibody-associated encephalitis with prominent cerebellar involvement (Fig. 5K and L).

**Discussion**

Using a mouse model prone to develop paraneoplastic neurological disorder, we explored the delicate balance between tumour immunity and autoimmunity. As expected, the anti-tumour T cell response was sufficient to reduce tumour growth. Similar to the human situation, anti-CTLA4 mAb, which antagonizes an inhibitory pathway in T cells, increased the efficacy of this anti-tumour immunity. Strikingly, however, this enhanced tumour control was obtained at the expense of autoimmune paraneoplastic neurological disorder. Actually, without anti-CTLA4 therapy, none of the studied animals developed cerebellar inflammation, whereas the use of anti-CTLA4 mAb led to cerebellar inflammation in 27/32 mice (84%).

Anti-CTLA4 therapy promotes anti-tumour immunity through several converging mechanisms in both mice and humans (Allison, 2015). In mice, anti-CTLA4 treatment
depletes Treg cells selectively from tumour via Fcγ receptor-expressing local macrophages, while the absolute numbers of effector T cells are increased (Simpson et al., 2013). In addition, CTLA4 blockade on effector T cells, notably on cytotoxic CD8+ T cells, appears essential for the enhanced anti-tumour response following anti-CTLA4 therapy. In that respect, human studies have shown that CTLA4 blockade therapy increases the amplitude of

Figure 5 Immune mechanisms of Purkinje cell loss in experimental and human paraneoplastic cerebellar degeneration. Cerebellum of wild-type (A) and L7-HA mice (B–J) 20 days after transfer of anti-HA specific T cells and anti-CTLA4 mAb therapy. (A and B) Calbindin staining shows marked loss of Purkinje cells in the L7-HA mice. Scale bars = 100 μm. (C) Double staining for CD8 (orange-brown) and calbindin (dark blue) shows the presence of large numbers of CD8+ T cells in the molecular layer of the cerebellum. Scale bar = 50 μm. (D) Double staining for CD8 and calbindin reveals cytotoxic T cells in contact with Purkinje cells. Scale bar = 20 μm. (E and F) Confocal fluorescence triple staining for Granzyme-B (red), CD8 (green) and calbindin (blue) shows CD8+ T cells in close apposition to Purkinje cells. Note the position of cytotoxic granules all facing the plasma membrane of the Purkinje cells. Scale bars = 10 μm. (G) Double staining for calbindin (blue) and β2-microglobulin (brown) showing MHC class I upregulation in the Purkinje cell. Scale bar = 25 μm. (H) Double staining for TUNEL (blue) and calbindin reveals degeneration of a Purkinje cell (arrowhead). Scale bar = 50 μm. (I) Presence of a caspase-3-positive cell with condensed nucleus in the Purkinje cell layer. Scale bar = 10 μm. (J) Microglial cells stained for Mac-3 enclosing a Purkinje cell. Scale bar = 25 μm. (K) Double staining for calbindin (blue) and CD8 (brown) shows severe loss of Purkinje cells and dendritic arborization in a patient with anti-Hu antibody-associated paraneoplastic neurological disorder. Scale bar = 200 μm. Arrowheads point at some remaining Purkinje cells. The inset shows a higher magnification of a CD8+ T cell (arrowhead) in tight apposition to a Purkinje cell. (L) Loss of Purkinje cells (arrowheads) can also be seen in a patient with anti-Ma2 antibody-associated paraneoplastic neurological disorder. Scale bar = 200 μm. Here the insets reveal multiple CD8+ T cells attacking Purkinje cells.
melanoma-specific CD8⁺ T cell responses against self-
antigens and, possibly, against tumour neoantigens
(Kvistborg et al., 2014; Van Allen et al., 2015).

Importantly, CTLA4 deficiency is associated with multi-
tissue lymphocytic infiltration and damage of likely autoim-
mune origin (Waterhouse et al., 1995; Ise et al., 2010). Here
again, CTLA4 maintains T cell tolerance by regulating the
pathogenicity of autoantigen-specific T cells by both effector
T cell autonomous effects and non-cell autonomous mechan-
isms, involving Treg cells (Wing et al., 2008; Ise et al., 2010).
Furthermore, patients with CTLA4 haploinsufficiency present
multi-tissue inflammation, including brain and cerebellar in-
volveinent (Kuehn et al., 2014; Schubert et al., 2014). Partly
mirroring these data, anti-CTLA4 therapy has been asso-
ciated with immune-related adverse events in ~60% of trea-
ted patients (Bertrand et al., 2015). In particular, CNS
autoimmunity is part of the spectrum of immune-related ad-
verse events of anti-CTLA4 therapy. Indeed, autoimmune
hypophysitis has been widely recognized as a frequent side
effect of ipilimumab therapy (Iwama et al., 2014; Bertrand
et al., 2015). Exacerbation of pre-existing autoimmune dis-
ease has also been reported in patients with advanced mel-
anoma treated with ipilimumab (Johnson et al., 2016). Whether de novo development of autoimmune side effects
is related to genetic susceptibility or to pre-existing serologic
evidence for autoantibodies is currently unknown.

In our mouse model, immune checkpoint therapy elicited
migration of antigen-specific T cells into the cerebellum and
subsequent killing of neurons, only when the antigen was
expressed in neurons. Our data strongly suggest that CD8⁺
T cells play a final effector role by specifically killing MHC
class I-expressing Purkinje cells in paraneoplastic cerebellar
deregeneration. Purkinje cells have indeed been reported to
express MHC class I molecules (McConnell et al., 2009), a
feature confirmed here both in vitro upon IFN-γ incubation
and in vivo. Based on these data and on the strong pro-
duction of IFN-γ by cerebellum-infiltrating CD8⁺ T cells, it
is tempting to speculate that a pathogenic feed-forward
loop takes place. This involves IFN-γ secretion by CD8⁺ T
cells upon local recognition of onconeural antigens that
further promotes MHC class I presentation by neurons, in
turn rendering them vulnerable to killing by antigen-specific
CD8⁺ T cells. A similar scenario is likely to play at human
paraneoplastic neurological disorders with autoantibodies
against intracellular neuronal antigens. Indeed, an IFN-γ
signature has been reported in the CSF of such patients
(Roberts et al., 2015). Additionally, CDR2-specific CD8⁺
T cells are present in the blood and CSF of patients with
paraneoplastic cerebellar degeneration (Albert et al., 1998,
2000) and they can kill CDR2-expressing target cells in an
HLA-restricted manner (Santomasso et al., 2007). Finally,
in situ evidence for direct killing of neurons by cytotoxic T
cells has been documented by converging studies (Bien et al.,
2012). Our still images from the experimental
model and from tissue of patients with paraneoplastic
neurological disorder point to a possible T cell cooperation
for killing, as some target neurons are contacted
simultaneously by two or more CD8⁺ T cells. This feature
was recently associated with high probability of target cell
death using dynamic two-photon imaging in experimental
models of viral infection (Halle et al., 2016).

Our data highlight the induction of paraneoplastic neuro-
logical disorder after therapeutic blockade of CTLA4, rais-
ing the concern that checkpoint inhibitors, by enhancing
anti-tumour immunity, may inadvertently increase the like-
lihood for paraneoplastic neurological disorders.
Paraneoplastic neurological disorder development has
been associated with a better control of the underlying
tumour, attributed to a partly efficient anti-tumour adap-
tive immunity (Pignon et al., 2013). In addition, the onco-
logic response to anti-CTLA4 therapy correlates with
immune-related adverse events (Bertrand et al., 2015).
Gynaecologic cancers and certain cancers of neuroectoder-
mal origin are particularly associated with paraneoplastic
neurological disorders. Consequently, the enhanced and
diversified anti-tumour T cell response promoted by anti-
CTLA4 therapy could lead to paraneoplastic neurological
disorder, in patients harbouring these types of cancers. As
suggested, based on theoretical grounds (Pignon et al.,
2013; Steinman, 2014), our experimental model argues
that immune checkpoint inhibitors may favour paraneo-
plastic neurological disorder development. Interestingly,
two cases of autoimmune encephalomyelitis developing
after the first injection of immune checkpoint inhibitors
have been recently reported (Williams et al., 2016). The
sero-prevalence of anti-neuronal autoantibodies can be as
high as 29% in patients with small cell lung cancer
(Pignon et al., 2013; Gozzard et al., 2015) and anti-neur-
onal antibodies are associated with T cells with the same
specificity (Albert et al., 2000). Therefore, a close monitor-
ing of neurological symptoms is warranted, at least in the
subgroup of patients with anti-neuronal autoantibodies,
when immune checkpoint inhibitors are being used.

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Supplementary material

Supplementary material is available at Brain online.

References


