CD4+ and CD8+ T cells are both needed to induce paraneoplastic neurological disease in a mouse model

Christina Gebauer, Béatrice Pignolet, Lidia Yshi, Emilie Mauré, Jan Bauer, and Roland Liblau

ABSTRACT

Paraneoplastic neurological disorders (PNDs) are rare human autoimmune diseases that mostly affect the central nervous system (CNS). They are triggered by an efficient immune response against a neural self-antigen that is ectopically expressed in neoplastic tumors. Due to this shared antigenic expression, the immune system reacts not only to tumor cells but also to neural cells resulting in neurological damage. Growing data point to a major role of cell-mediated immunity in PNDs associated to autoantibodies against intracellular proteins. However, its precise contribution in the pathogenesis remains unclear. In this context, our study aimed at investigating the impact of anti-tumor cellular immune responses in the development of PND. To this end, we developed an animal model mimicking PND. We used a tumor cell line expressing the hemagglutinin (HA) of influenza virus to induce an anti-tumor response in CamK-HA mice, which express HA in CNS neurons. To promote and track the T cell response against the HA antigen, naive HA-specific CD8+ and/or CD4+ T cells, originating from TCR-transgenic animals, were transferred into these mice. We demonstrate that HA-expressing tumors, but not control tumors, induce in vivo activation, proliferation and differentiation of naive HA-specific CD4+ and CD8+ T cells into effector cells. Moreover, both T cell subsets were needed to control tumor growth and induce CNS inflammation in CamK-HA mice. Thus, this new mouse model provides further insight into the cellular mechanisms whereby a potent anti-tumor immunity triggers a cancer-associated autoimmune disease, and may therefore help to develop new therapeutic strategies against PND.

Introduction

Paraneoplastic neurological disorders (PNDs) are neurological conditions that develop in cancer patients in whom an efficient anti-tumoral immune response causes secondary autoimmunity in the nervous system. PNDs do not reflect direct tumor invasion of neuronal tissues, but result from an immune response directed against onconeural antigens, defined as being ectopically expressed by tumor cells but physiologically expressed by neural cells.1 PNDs are the best-documented examples showing a direct link between naturally occurring immunity against malignancy and autoimmunity.2 However, the immune actors involved in these 2 phenomena remain to be determined.

Different PNDs have been identified, characterized by the tumor-associated antigen targeted by the immune system and, therefore, the neural cell type affected. As a consequence, patients with PNDs exhibit different neurological manifestations. Nevertheless, PNDs share some common characteristics. First, in most cases the neoplasm is detected only after the neurological manifestations of PND can be of small size or even fully controlled.3 Second, patients with PNDs harbor high titers of antibodies against onconeural antigens in their serum and cerebrospinal fluid (CSF), whereas patients without PND bearing the same malignancy do not. Therefore, detection of these autoantibodies carries a high diagnostic value. Not only they define the type of PND but also they help identify the underlying tumor.4,5 These autoantibodies can actually play a pathogenic role in PNDs in which the target antigens are extracellular molecules, such as neurotransmitter receptors or voltage-gated calcium channels.6,7 In contrast, for PNDs associated with autoantibodies targeting intracellular antigens, such as Ma2, Cdr2 and HuD, transfer of antibodies fails to elicit disease in experimental animal models.8 A major role for the T cell-mediated immune response has therefore been suggested. Indeed, in those patients,
Autoantigen-specific T cells could be detected in blood or CSF. Moreover, infiltration of the CNS parenchyma by CD8+ T cells is a salient feature, with occasional identification of cytotoxic T cells in close proximity to damaged neurons. Interestingly, oligoclonal cytotoxic T cells have been identified in both brain and tumor of patients with the anti-Hu syndrome, suggesting an antigen-driven clonal expansion. These findings are strengthened by the fact that therapeutic strategies acting on the humoral immune response showed no benefit for patients with PNDs associated with autoantibodies against intracellular antigens. To date, the treatment of these PNDs remains very challenging. To develop effective therapeutic strategies, it is crucial to decipher the pathophysiological mechanisms underlying PND. To that goal, an animal model mimicking PND would be valuable. So far, attempts to model PND by immunization with protein or DNA, or adoptive transfer of antigen-specific T cells failed to reproduce signs or pathological features of PND. In this study, we developed an original mouse model in which most CNS neurons express the same model antigen as an implanted tumor. In these mice, T cells specific for this antigen are activated in the periphery by the tumor antigen and further migrate into the CNS causing inflammation and tissue damage. Using this new in vivo model, we investigate the contribution of CD4+ and CD8+ T cells in the course of the disease as well as their functional and phenotypic characteristics.

Results

Collaboration of HA-specific CD4+ and CD8+ T cells is needed to control growth of HA-expressing tumors

As the first step to model PND in mice, a neo-self antigen, the hemagglutinin of influenza virus (HA), was introduced in a transplantable tumor, the 4T1 mouse mammary carcinoma. The resulting 4T1-HA cells express high levels of MHC class I molecules, but differ from 4T1 cells with respect to their expression of HA (Supplementary Fig. 1A). Both types of tumors grew similarly and were uncontrolled in the absence of adoptively transferred HA-specific T cells (Supplementary Fig. 1B).

To elicit an anti-tumor T cell response, mice implanted with the 4T1-HA tumor or its parental line, received naive HA-specific CD4+ and/or CD8+ T cells isolated from TCR-transgenic mice. The CD45.1 congenic marker expressed by the transferred HA-specific T cells allows distinguishing them from the endogenous T cells of the recipient animals.

We first investigated the capacity of the 4T1-HA tumor to activate naive HA-specific T cells. Thus, CFSE-labeled CD45.1+ CD4+ T cells and CellTrace Violet-labeled CD45.1+ CD8+ T cells were co-injected into syngeneic recipient mice, previously implanted with either 4T1 or 4T1-HA tumor. Six days post-transfer, in vivo proliferation of both HA-specific CD4+ and CD8+ T cells was evidenced by dilution of the fluorescent dyes in 4T1-HA-bearing mice, whereas proliferation of HA-specific T cells was weak in mice implanted with 4T1 tumor (Fig 1 A & B). A high proportion of cycling HA-specific CD4+ and CD8+ T cells produced IFN-γ and TNF-α upon ex vivo stimulation, indicating a type 1 polarization, following in vivo activation by the HA-expressing tumor (Fig 1 A & B). In contrast, HA-specific T cells barely acquired effector functions in 4T1-bearing mice (Fig 1 A & B).

PND are often associated with a partially efficient anti-tumor immune response. We, therefore, investigated the ability of the 2 HA-specific T cell subsets, alone or in combination, to control the 4T1-HA tumor. Tumor growth was assessed in recipient mice implanted with 4T1-HA cells and transferred the same day with naïve HA-specific CD8+ T cells and/or naïve HA-specific CD4+ T cells. HA-specific CD8+ T cells partially controlled the tumor whereas HA-specific CD4+ T cells had no detectable effect. Interestingly, the co-injection of naïve HA-specific CD8+ and CD4+ T cells allowed tumor control (Fig 1C). Indeed, more than 40% of mice injected with both types of T cells remained free of tumor (Fig 1C). No such tumor control was observed in mice bearing the parental 4T1 tumor (data not shown).

Tumor control by HA-specific CD4+ and CD8+ T cells is associated with a neurological phenotype in CamK-HA mice

To model the human PND situation in which the same antigen is expressed by both tumor cells and CNS neurons, we made use of CamK-HA mice that express HA in most CNS neurons. Interestingly, significant weight loss was observed in CamK-HA mice implanted with 4T1-HA tumor following transfer of naïve HA-specific CD4+ T cells and CD8+ T cells. Seventy two percent of the CamK-HA animals showed over 5% loss of their initial weight (Fig 2A). Ten percent of CamK-HA mice also exhibited obvious behavioral manifestations associating shiver, hypoactivity, and limp tail (Supplementary Video 1), which led to death or sacrifice of 7% of these mice. The disease was however transient since most surviving mice recovered their initial weight and motor abilities within 3 weeks (data not shown).

CamK-HA mice bearing the parental 4T1 tumor or wild-type (WT) mice implanted with the 4T1-HA tumor were devoid of such clinical manifestations following transfer of HA-specific CD4+ and CD8+ T cells (Fig 2A). This excludes tumor spread as disease cause and indicates that anti-tumor T-cell response was not sufficient to elicit neurological manifestations in the absence of expression of ‘oncneural’ HA antigen in the CNS. The antigen has indeed to be present in both tumor and CNS. Intriguingly, in few CamK-HA mice (n = 3) implanted with 4T1-HA and transferred with both HA-specific CD4+ and CD8+ T cells, upper gastro-intestinal occlusion was observed without signs of tumor dissemination or external compression. Histology showed local granulocyte infiltration but very few T cells (Supplementary Fig. 2A and 2C). Sensitive qRT-PCR did not reveal HA expression in the stomach or small intestine (Supplementary Fig. 2B), arguing against ectopic expression of the HA transgene and subsequent autoimmune reaction involving the gastro-intestinal tract.

To assess the individual contribution of CD4+ and CD8+ T cells in the phenotype, 4T1-HA-bearing CamK-HA mice
received either no T cells, HA-specific CD4+ T cells, HA-specific CD4+ T cells, or both. Only CamK-HA mice injected with both types of T cells developed weight loss and neurological signs (Fig 2B).

Collectively, the data indicate that, in CamK-HA mice bearing the 4T1-HA tumor, the collaboration of HA-specific CD4+ and CD8+ T cells is necessary for a successful anti-tumor immune response as well as for the induction of neurological signs. Notably, the presence of the ‘onconeural’ HA antigen in both tumor and brain was required for the neurological phenotype, pointing to its antigen-specific basis.

The neurological phenotype in CamK-HA mice is associated with CNS inflammation

We then assessed whether the clinical manifestations reflected CNS inflammation and neuronal damage. To this end we analyzed histologically the CNS of the affected CamK-HA mice at day 8 (disease onset) and day 15 (peak) after implantation of the HA-expressing tumor. Widespread parenchymal T-cell infiltration was detected only in the group expressing HA in both tumor and CNS neurons. T cells were more abundant in the spinal cord, and the hypothalamus (Fig 3A, C), but

Figure 1. HA-specific CD4+ and CD8+ T cells are activated by, and control the growth of, a HA-expressing tumor. Adoptive transfer of 10^7 CFSE-stained HA-specific CD45.1+ CD25-CD62L+ CD4+ T cells and 10^7 CellTrace Violet (CTV)-stained HA-specific CD45.1+CD62L+ CD8+ T cells into wild-type (WT) mice bearing either the 4T1-HA or the 4T1 tumor. At day 6, spleen and draining lymph node cells were stimulated with PMA/ionomycin for 4 hours. FACS analysis was performed to assess proliferation/fluorescent dye dilution and production of IFN-γ and TNF-α by the transferred CD45.1+ T cells. (A) Representative FACS plots of splenocytes from a mouse carrying either the 4T1-HA (left) or 4T1 (right) tumor. (B) Frequency of IFN-γ-producing CD45.1+ CD4+ or CD45.1+ CD8+ T cells in the spleen. Pooled data from 3 independent experiments, data represent the mean ± SEM of 8 mice with 4T1-HA and 7 mice with 4T1 tumors. Mann-Whitney, **p < 0.01. (C) CamK-HA bearing the 4T1-HA tumor received either no T cells, naive HA-specific CD45.1+CD25-CD62L+ CD4+ T cells (10^7), naive HA-specific CD45.1+CD62L+ CD8+ T cells (10^7), or both types of T cells (10^7 each). Pooled data from 3 independent experiments are shown. Left: tumor size, each value represents the mean ± SEM of the group. Two-way ANOVA, ****p < 0.0001. Right: percentage of tumor-free animals. Log-rank (Mantel-Cox) test, n.s = not significant, ****p < 0.0001.
infiltrated also the cortex, cerebellum, hippocampus and the brainstem (data not shown). Some of the infiltrates could be identified as CD8+ T cells (Fig 3B, D, E, H). Between 4.6 and 16.9% of T cells were localized in close apposition to neurons depending on the CNS region. In some areas, such as the hypothalamus, cells producing granzyme B were found in close proximity to neurons (Fig 3F). Concomitantly, loss of neurons (Fig 3D) and caspase 3 activity (Fig 3I, J) as a sign of neuronal apoptosis were detected in these areas. Mac-3 staining revealed intense macrophage/microglia activation at sites of T-cell infiltration (Fig 3G).

We additionally characterized the immune cells penetrating the CNS at an early phase using flow cytometry. A significant increase in infiltrating T cells and blood-derived myeloid (CD11bhighCD45high) cells was observed in 4T1-HA bearing CamK-HA mice (Fig 4A, B). The CNS-infiltrating T cells are composed of 22 ± 3.3% of transferred CD45.1+ HA-specific T cells and 78 ± 4.1% of endogenous CD45.1- T cells (Fig 4C). Notably, among CD45.1+ HA-specific T cells 81.6 ± 4.1% are CD8+ T cells. In contrast, slightly more CD4+ T cells (58.4 ± 5.8%) than CD8+ T cells (41.6 ± 5.8%) were present among endogenous infiltrating T cells (Fig 4C). These data indicate a preferential recruitment of HA-specific CD8+ T cells into the CNS and strongly suggest that they represent the major effector cells driving the inflammation in this mouse model. Concomitantly, upregulation of MHC class II molecules on microglia (CD45intCD11bhigh) was observed in CamK-HA mice bearing the 4T1-HA tumor (Fig 4D). At a later time point (day 21), HA-specific T cells were virtually absent from the CNS (Supplementary Fig. 3A, B), documenting the monophasic course of the disease. To investigate whether this monophasic course of the disease is due to the lack of antigen in the periphery after efficient tumor control, PND mice were re-injected at the peak of the disease with the 4T1-HA tumor alone or together with naïve HA-specific CD4+ and CD8+ T cells. Neither clinical manifestations nor increase in CNS-infiltrating T cells could be observed after the second exposure to HA antigen and HA-specific T cells in these mice (Supplementary Fig. 3A–D).

Phenotype of infiltrating T cells in the CNS vs. tumor

We next investigated in more depth the phenotype of T cells in CNS, tumor and spleen by flow cytometry. Among transferred CD8+ and CD4+ T cells, about 40–50% express IFN-γ and TNF-α in the CNS. No significant difference could be detected for transferred CD8+ T cells between organs (Fig 5). In contrast, a higher proportion of cells producing both IFN-γ- and TNF-α-producing cells were present in the CNS compared with the spleen for transferred CD4+ T cells (Fig 5). The frequency of GM-CSF- or IL-17A-producing T cells was low in all organs (Fig 5). Nevertheless, among transferred CD8+ and CD4+ T cells, there was a greater accumulation of GM-CSF and IL-17A-producing cells in the CNS and in the tumor compared with their splenic counterparts. The endogenous T cells exhibited the same functional properties. However, the frequency of endogenous CD4+ T cells producing IFN-γ and TNF-α was decreased in the tumor as compared to the CNS (Fig 5). These results indicate a preferential accumulation of type-1 polarized T cells in the CNS whereas an enrichment of type-17 polarized T cells was found in the tumor.

Analysis of the surface markers CD25, CD44, CD62L, KLRG1 and CD127 revealed differences in the phenotype of CNS-infiltrating T cells vs. both tumor-infiltrating and splenic T cells. Indeed, the frequency of transferred and endogenous effector CD8+CD44highCD62Llow T cells was higher in CNS than in tumor or spleen. In contrast, no significant difference could be found for transferred and endogenous CD4+ effector T cells between CNS and tumor (Fig 6). Higher expression of CD25 was also detected on CNS and tumor-infiltrating T cells than on their splenic counterparts (Supplementary Fig. 4). Furthermore, we found a higher frequency of central memory CD44highCD62Lhigh HA-specific and endogenous CD8+ T cells in the spleen.
than in the CNS or tumor (Fig 6). The proportion of short-lived effector CD8+ T cells (SLECs) that were shown to be KLRG1<sup>high</sup>CD127<sup>low</sup> was low in all 3 organs. In contrast to HA-specific CD8<sup>+</sup> T cells, endogenous CD8<sup>+</sup> T cells displayed a higher frequency of SLECs in CNS and tumor than in the spleen. Terminally differentiated and exhausted CD4<sup>+</sup> T cells are also defined as KLRG1<sup>high</sup>CD127<sup>low</sup>. In our model, transferred CD4<sup>+</sup> T cells with this phenotype were mainly present in the CNS in contrast to endogenous CD4<sup>+</sup> T cells that were also present at the tumor site. Collectively, CNS-infiltrating T cells showed a higher frequency of activated/effector T cells than cells residing in the tumor and the spleen. The large proportion of KLRG1<sup>high</sup>CD127<sup>low</sup> CD4<sup>+</sup> T cells can be an indicator for exhaustion. Interestingly, a large proportion of CD8<sup>+</sup> T cells in the spleen showed already a memory phenotype.

**Treatment**

We next aimed to interfere in vivo with T-cell migration into the CNS while preserving an efficient anti-tumor immune response. More specifically, we tested an approach relying on the blocking anti-α4 integrin mAb (PS/2), which prevents the migration of the encephalitogenic leukocytes across the blood-brain-barrier through its binding to the VLA-4/α4β1 integrin. However, treatment started at day 6 did not significantly alleviate clinical signs (Fig 7A) or CNS inflammation (Fig 7B). Interestingly, the proportion HA-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells expressing α4 integrin (CD49d) in the spleen and the CNS is limited on our model (CD4<sup>+</sup> T cells ranging from 0 to 3%; CD8<sup>+</sup> T cells ranging from 2.3 to 16.2%). These data suggest that T cells primed in the tumor microenvironment exhibit migratory properties different from those of autoreactive T cells generated during EAE.

**Discussion**

In this present study, an animal model of PND has been generated recapitulating the human pathology characterized by an efficient tumor control and CNS autoimmunity. Indeed, T cells were able to both control the tumor growth and infiltrate the CNS causing neuroinflammation, with prominent activation of...
infiltrating CD4+ T cells produces IFN-γ and TNF-α, as observed in PND patients. Another group elicited modest experimental CNS inflammation by adoptive transfer of Th1 effector CD4+ T cells specific for the neuronal protein PNMA1 that belongs to the paraneoplastic Ma family of onconeural protein in humans. However, no clinical phenotype was observed.

In our model, antigen-specific CD4+ T cells alone were unable to induce CNS inflammation or to control the underlying tumor. In contrast, CD8+ T cells could partially control the tumor but were not sufficient to elicit T-cell infiltration into the CNS. Our observations are consistent with the data from Marzo and colleagues. They demonstrated that the induction of CTL function is not sufficient for tumor rejection. The help of CD4+ T cells is needed to maintain the CTL pool, its functions and its recruitment to the tumor.

In our study, we strongly suggest that CD4+ T cells involved in PND exhibit a Th1 phenotype as the majority of CNS infiltrating CD4+ T cells produces IFN-γ and TNF-α, as observed in PND patients. Another group elicited modest experimental CNS inflammation by adoptive transfer of Th1 effector CD4+ T cells specific for the neuronal protein PNMA1 that belongs to the paraneoplastic Ma family of onconeural protein in humans. However, no clinical phenotype was observed. In our model, antigen-specific CD4+ T cells alone were unable to induce CNS inflammation or to control the underlying tumor. In contrast, CD8+ T cells could partially control the tumor but were not sufficient to elicit T-cell infiltration into the CNS. Our observations are consistent with the data from Marzo and colleagues. They demonstrated that the induction of CTL function is not sufficient for tumor rejection. The help of CD4+ T cells is needed to maintain the CTL pool, its functions and its recruitment to the tumor.
functions. A study on autoimmune diabetes provided similar results. In a transgenic mouse model in which OVA is expressed in the pancreatic β cells as a model self-antigen, anti- gen-specific CD4+ T cells improved the survival of CTLs, thus, extending the time to perform their effector functions. Furthermore, it was shown that CD4+ T cell help is also important to avoid the induction of CTL tolerance. We therefore suggest that CD4+ T cells play a critical role in the disease course of PND in helping CTLs to acquire effector functions and to increase their survival. The maintenance of a pool

Figure 5. A large proportion of CNS-infiltrating T cells exhibits a type-1 phenotype. Adoptive transfer of naïve HA-specific CD45.1+ CD8+ T cells and CD8+ T cells into CamK-HA mice bearing the 4T1-HA tumor. FACS analysis was performed on T cells from CNS, tumor, and spleen following PMA/ionomycin stimulation between day 10 and 14. The proportions of transferred CD45.1+ CD8+ (upper left), transferred CD45.1+ CD4+ (upper right), endogenous CD45.1- CD8+ (lower left) and endogenous CD45.1- CD4+ (lower right) T cells in the CNS (n = 10), tumor (n = 5) and spleen (n = 10) that express IFN-γ and TNF-α, GM-CSF or IL-17A are plotted. Pooled data from 3 independent experiments, one value is from a pool of 4 mice for each organ, data represent the mean ± SEM. Kruskal-Wallis, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

Figure 6. CNS infiltrating T cells show an effector phenotype. CamK-HA mice were induced to develop PND as described in Figs. 4 and 5. FACS analysis of the CNS (n = 8), tumor (n = 3) and spleen (n = 8) at the peak of the disease. Comparison of the proportion of congenic CD45.1+ CD8+ (upper left), congenic CD45.1+ CD4+ (upper right), endogenous CD45.1- CD8+ (lower left) and endogenous CD45.1- CD4+ (lower right) T cells in the CNS, tumor and spleen. The marker CD44, CD62L, KLRG1 and CD127 were assessed to identify effector T cells (CD44highCD62Llow), central memory T cells (CD44highCD62Lhigh) and for CD8+ T cells: short-lived effector cells and for CD4+ T cells: terminally differentiated and exhausted cells (KLRG1highCD127low). Pooled data (mean ± SEM) from 2 independent experiments, each value derives from a pool of 4 mice for each organ. Kruskal-Wallis, * p < 0.05, ** p < 0.01.
of autoreactive CTLs could therefore break tolerance and may promote migration into the CNS.

In the CNS, over 80% of infiltrating HA-specific T cells were CD8+ T cells, suggesting that these cells are the main immune component driving CNS inflammation in our model. This is consistent with the findings in human post-mortem samples, in which histological analysis of brain samples revealed CD8+ T cells producing granzyme B in close proximity to neurons. We could reproduce these findings in our model underlying the effector role of CD8+ T cells in PND, inferring that CD8- T cells are the effector T cells that cause neuroinflammation, with production of cytokines and cytolytic granules toward neurons. In contrast to other PND animal models, we could elicit a clinical phenotype that, in rare cases, led to occlusion of the upper gastro-intestinal tract. This manifestation was previously reported in PND patients.

In our model, 70% of the mice underwent significant weight loss and showed CNS inflammation. However, most of the mice recovered by day 21. The monophasic course of the disease was not due to the lack of antigen in the periphery, as re-injection of the tumor in mice that developed PND could not sustain the CNS inflammation. Similar observations were made upon a second transfer of HA-specific CD4+ and CD8+ T cells together with the re-inoculation of the HA-expressing tumor. This suggests that the monophasic nature of the disease likely results from exhaustion of HA-specific effector T cells secondary to persistent antigen exposure in the CNS probably associated to immunoregulatory pathways mediated, among others, by T regulatory cells. The observation of a large proportion of KLRG1highCD127low T cells in CNS and tumor argues in favor of the involvement of T cell exhaustion. However, it is unclear if the exhaustion of HA-specific T cells occurs in the tumor, CNS or both.

This new experimental model of PND could be used as a preclinical model to test molecules known to prevent CNS inflammation. One of the major challenging tasks was to not interfere with the efficient anti-tumoral immunity. We tested a therapy already marketed for multiple sclerosis; namely the monoclonal antibody against α4 integrin. However, even though we did not interfere with anti-tumoral immunity, this approach or the settings we used were not able to prevent CNS inflammation. Therefore, further studies have to be performed to develop an efficient treatment for PND.

This animal model provides an unprecedented resolution of the inflammatory response that causes PND and will permit the pre-clinical validation of novel therapeutic approaches.

Materials and methods

Mice

CD45.1 mice, CamK-HA mice, CL4-TCR and 6.5-TCR mice have been described previously. All mice were bred on a [BALB/c × C57BL/6] F1 background. Mice were kept under specific pathogen-free conditions. All experimental procedures were conducted according to EU guidelines and have been approved by the regional ethics committee (Ref: MP/09/23/03/14.09.2012, Comité d’éthique pour l’expérimentation animale Midi-Pyrénées, approved 14.09.2012)

Tumor cell lines

The 4T1 tumor cell line, derived from a BALB/c spontaneous mammary breast carcinoma, and its 4T1-HA derivative expressing hemagglutinin (HA) of the influenza virus (provided by D. Klatzman, Hôpital Pitié Salpêtrière), were cultured in DMEM GlutaMAX™-1 Medium (Invitrogen) supplemented with 10% fetal calf serum (PAA Laboratories), 1% penicillin-streptomycin (Invitrogen), 1% HEPES buffer solution 1 M (PAA Laboratories) and 0.1% β-mercaptoethanol (Invitrogen). For in vivo experiments, 105 4T1 or 4T1-HA cells diluted in PBS were injected s.c. into the flank of the mice (100μl/mouse).

Naïve HA-specific T cells

Naïve HA-specific CD4+ T cells and CD8+ T cells were isolated from 6.5-TCR and CL4-TCR mice, respectively. Lymph node and spleen single cell suspensions underwent negative selection using Dynabeads (Invitrogen) (for naïve CD4+ T cells, the PC61 anti-
CD25 mAb was added), followed by a positive selection step using CD62L microbeads (Miltenyi). Naïve CD4+ and/or CD8+ T cells (10^7 cells each) were diluted in 200 μl PBS and injected i.v. into recipient mice. For some experiments purified T cells where stained with CFSE and CellTrace Violet (Invitrogen) before injection. The purity of the T cell subsets was verified by flow cytometry and ranged from 89.3% to 96.9%.

Clinical follow-up and treatment

Mice were assessed daily for neurological signs and weight loss. Tumor size was determined every other day by measuring perpendicular diameters, using a Vernier caliper and expressed in mm^2 (w × L).

The anti-α4 integrin antibody (PS-2 clone, kindly provided by B. Engelhardt, Bern University) was injected i.v. 250 μg/injection/mouse at the indicated time points.

Immune cell suspension from tissues

Mice were sacrificed and their spleens and draining lymph nodes were harvested at the indicated time points. The tissues were smashed and red blood cells were lysed using a lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, 0.1 mM Na₂EDTA, 0.5 M dH₂O, adjusted to pH 7.2).

To isolate inflammatory cells from the CNS, mice were perfused with PBS. Brains and spinal cords were dissected, cut in 2 mm pieces and incubated in collagenase A (Roche, 2mg/ml) in RPMI Medium 1640 (Invitrogen), DNase I (Roche, 0.2mg/ml) and N-a-Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK, Sigma, 0.02mg/ml) for 30–40 minutes at 37°C. After a washing step, mononuclear cells were collected following a percoll gradient separation.

For isolation of tumor-infiltrating cells, the tumor was dissected, cut in 2 mm pieces and incubated in collagenase A (Roche, 2mg/ml) in RPMI Medium 1640, DNase I (0.2mg/ml) for 30 minutes at 37°C. After a washing step, the tumors were further smashed, filtered and centrifuged.

FACS analysis

CNS- and tumor-infiltrating cells as well as spleen and lymph node cells were stained using the following antibodies: anti-Thyl.2 (53–2.1, BD Bioscience), anti-CD45.2 (104, BD Bioscience), anti-CD45.1 (A20, BioLegend), anti-CD4 (30–Fl1, BD Bioscience), anti-CD8β (YTS156.7.7, BioLegend), anti-CD4 (RM4.5, BD Bioscience), anti-CD11b (M1/70, eBioscience), anti-MHC-II (M5/114.15.2, eBioscience), anti-CD44 (IM7, eBioscience), anti-CD25 (PC61.5, eBioscience), anti-Ly6c (AL-21, BioLegend), anti-KLRG1 (LFA/KLRG1, BioLegend), anti-CD127 (SB/199, BD Bioscience), anti-IFNγ (XM1G1.2, BD Bioscience), anti-TNFα (MP6-XT22, BD Bioscience), anti-GM-CSF (MP1–22E9, BD Bioscience), and anti-IL-17A (TC11–18H10, BD Bioscience).

In vivo proliferation of CD45.1-congenic HA-specific T cells in secondary lymphoid organs was evaluated through dilution of CFSE and CellTrace Violet. Additionally, after stimulation using PMA (Sigma, 1μl/mL), ionomycin (Sigma, 2μl/mL) and Golgi plug (GE Healthcare, 2μl/mL) for 4 hours, cells were stained with anti-IFN-γ (XM1G1.2) and anti-TNF-α (MP6-XT22) antibodies.

Data were collected on a LSRII or LSRII Fortessa (Becton Dickinson) and analyzed with FlowJo software (Tree Star).

Neuropathology

Mice were perfused with PBS followed by 4% PFA. Tissues were removed and embedded in paraffin. Immunohistochemical staining was performed on 5-μm-thick serial sections using the following Abs: anti-CD3 (1:200, SP7, Zytomed), anti-Mac3 (1:200, BD), anti-Granzyme B (1:25, Santa Cruz), anti-CD8 (1:5000, Ebiosciences), anti-CD4 (1:250, Ebiosciences). The secondary antibodies used were biotinylated anti-rabbit and anti-rat antibodies (Jackson) followed by avidin-peroxidase (Sigma). Stainings were developed with 3,3′-diaminobenzidine (DAB).

We counted the number of T cells in the gray matter of the spinal cord, the hippocampus and the hypothalamus, and quantified the proportion of T cells in direct contact to a neuron.

RT-PCR

To assess HA expression, total RNA was extracted with RNeasy Mini Kit (Qiagen), followed by reverse transcription (SuperScript III). HA mRNA expression was normalized to HPRT mRNA. The following primers were used to amplify HA mRNA (Forward 5’-AACTCTTGGGCGTGCTTCTTCA-3’, Reverse 5’-GATAAGTGACTTGGGCTGCG-3’), and HPRT (Forward 5’TGGTTAAGCAGTACAGGCCCCCA-3’, Reverse 5’-AGGT CCTTTACCAGCAAGCT-3’).

Statistical analyses

Differences between 2 sets of data were evaluated by a Mann-Whitney test. Multiple comparison data were analyzed using the Kruskal-Wallis test (post-test Dunn). For tumor size, data were analyzed using the 2-way ANOVA (post test Bonferroni). Survival was analyzed by Log-Rank test (Mantel-Cox). The analyses were performed on Prism 6 (GraphPad Software). Groups were considered statistically different at a p-value of < 0.05. All data are presented as the mean ± SEM.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions
Christina Gebauer: experimental design, performed the experiments, bibliography, writing manuscript
Beatrice Pignolet: experimental design, performed the experiments, bibliography, writing manuscript
Lidia Yshii: helped with experiments and reviewed the manuscript
Emilie Mauřé: helped with experiments
Jan Bauer: performed neuropathology experiments and reviewed the manuscript
Roland Liblau: experimental design, interpretation of the data, writing manuscript

ORCID
Jan Bauer (http://orcid.org/0000-0001-5802-8047)

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