Cutting Edge: Neuronal Recognition by CD8 T Cells Elicits Central Diabetes Insipidus

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An increasing number of neurologic diseases is associated with autoimmunity. The immune effectors contributing to the pathogenesis of such diseases are often unclear. To explore whether self-reactive CD8 T cells could attack CNS neurons in vivo, we generated a mouse model in which the influenza virus hemagglutinin (HA) is expressed specifically in CNS neurons. Transfer of cytotoxic anti-HA CD8 T cells induced an acute but reversible encephalomyelitis in HA-expressing recipient mice. Unexpectedly, diabetes insipidus developed in surviving animals. This robust phenotype was associated with preferential accumulation of cytotoxic CD8 T cells in the hypothalamus, up-regulation of MHC class I molecules, and destruction of vasopressin-expressing neurons. IFN-γ production by the pathogenic CD8 T cells was necessary for MHC class I upregulation by hypothalamic neurons and their destruction. This novel mouse model, in combination with related human data, supports the concept that autoreactive CD8 T cells can trigger central diabetes insipidus. The Journal of Immunology, 2012, 188: 4731–4735.

Despite its relative “immune privilege,” the CNS is the site of an adaptive immune reaction in numerous diseases due to infections, tumors, neurodegeneration, or autoimmunity (1, 2). CNS autoimmune diseases can target different cell types, including oligodendrocytes, as is highly suspected in multiple sclerosis, astrocytes, as recently recognized in neuromyelitis optica, or even neurons (3). Indeed, an increasing number of CNS diseases is associated with, or caused by, autoimmunity against widespread or discrete neuronal populations. For example, pathologies involving neurons localized in many anatomical structures include paraneoplastic encephalomyelitis associated with anti-Hu Abs (4) and anti–N-methyl-D-aspartate receptor Ab-associated encephalitis (5). However, the antigenic targets of the autoimmune process can be much more restricted as found in narcolepsy, which affects selectively hypocretin-producing hypothalamic neurons, or in paraneoplastic cerebellar degeneration in which Purkinje neurons are specifically destroyed (4). Although the pathogenic role of autoantibodies that recognize surface receptors has been strongly suspected in some clinical situations (5), the immune effectors leading to tissue damage remain speculative in most cases. However, given that neurons can be induced to express MHC class I molecules, a role for CD8 T cells specific for neuronal self-antigens is suspected (3). A role for CD8 T cells has recently emerged in multiple sclerosis as CD8 T cells are clonally expanded within active lesions. Notably, the density of CD8 T cells correlates with the extent of axonal damage in lesions, supporting the notion that these cells actively contribute to the observed tissue damage (6).

To address this issue, we developed a novel mouse model expressing a “neo-self” Ag in CNS neurons of the CNS. We show that transfer of “neo-self”–specific cytotoxic CD8 T cells (CTLs) induces a severe but transient encephalomyelitis in these mice. Intriguingly, all the surviving mice develop chronic central diabetes insipidus because of the specific killing of hypothalamic magnocellular vasopressin-expressing neurons. We provide mechanistic insight for the unique vulnerability of these neurons to CD8 T cell-mediated killing owing to their remarkable propensity to upregulate MHC class I molecules in response to IFN-γ.

**Materials and Methods**

**Mice**

The CamK-iCre (7), the Rosa26^{tm}HAI/Lib, and the CL4-TCR mice have been described previously (8). All mice were back-crossed at least six times on the BALB/c background. Mice were kept under specific pathogen-free conditions. All experimental procedures were approved by the local ethics committee.

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Abbreviations used in this article: AVP, arginine vasopressin; GrB, granzyme B; HA, hemagglutinin.

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PCR and nested RT-PCR
Cre-mediated recombination of the Rosa26<sup>tm(HA)1Lib</sup> locus was assessed by PCR on genomic DNA extracted from different organs using primers P1 5'-GTCGCTCTGAGTTTATCATGAGAAGC-3' and P2 5'-GTCGCTCA-TCTCAAGCTGAACTGC-3'. To analyze hemagglutinin (HA) expression, RNA was extracted using TRIzol, and reverse transcription was performed with SuperScript III (Invitrogen) using priming with random hexamers. This was followed by a nested PCR approach using first the P1 and P2 primers and then the P3 5'-CTGATCTGAACTCCAGCTC-3' and P4 5'-CTGCAGGATTCGATATCA AGC-3' primers.

**FACS analysis**
CD8<sup>+</sup> T cell cultures were stained using anti-CD8α (53-6.7), anti-Vβ8 (F23.1), anti–IFN-γ (XMG1.2), anti–TNF-α (MP6-XT22), and anti-granzyme B (BD Pharmingen) Abs. CNS-infiltrating cells were stained using anti-CD45 (30-F11; BD Pharmingen), anti-Thy1.2 (53-2.1; eBioscience), anti-CD4 (RM4-5; BD Pharmingen), and anti-Vβ8 (F23.1) Abs. Data were collected on an LSRII or a FACSCalibur (Becton Dickinson) and analyzed with FlowJo software (Tree Star).

**In vitro differentiation, GFP transduction, and adoptive transfer of HA-specific cytotoxic CD8<sup>+</sup> T cells**
HA-specific CTLs and GFP-transduced CD8<sup>+</sup> T cells were generated as described (8). Between 94.5 and 98.7% of the transferred cells were CD8<sup>+</sup> T cells expressing the transgenic TCRβ-chain (Supplemental Fig. 1A). At day 5, living cytotoxic CD8<sup>+</sup> T cells were injected i.v. in recipient mice.

**Rota-Rod test**
Motor coordination was assessed with a Rota-Rod machine (Bioseb, Vitrolles, France). The rotation of the rod was accelerated from 4 to 40 rpm over 10 min. After a 2-d habituation period, each animal was assessed two to three times per week after T cell transfer. Every time, four trials were performed. The latency to fall from the rod was measured, and the longest time out of the four trials was recorded.

**Measurement of fluid intake and urine output**
Mice were individually housed in metabolic cages (Tecniplast, Lyon, France) for 12 h (8 AM to 8 PM) with free access to water to measure the basal water intake and urine output. When noted, the arginine vasopressin (AVP) antiserum (RM4-5; BD Pharmingen) and for each mouse the peak value was determined. Results were analyzed using the Student t test, Mann–Whitney U test, or log-rank test.

**Histopathology**
Mice were perfused, and tissues were removed and either fixed in OCT and embedded in paraffin. Immunohistochemically stained sections containing 5- or 20-μm-thick serial sections were performed using the following Abs: mouse anti-HA antibody (Minetrix) was injected s.c. (2 μg/kg body weight) at the beginning of the measurement and 4 and 8 h later. Water intake and urine output were measured during 12 h. Each mouse was tested twice with and without desmopressin injection with a rest of at least 2 d between the two experiments, and each value is the mean of the two measurements.

**Measurement of plasmonic vasopressin**
Plasmonic vasopressin concentration was determined using the Arg<sup>8</sup>-Vasopressin EIA Kit (Assay Designs, Ann Arbor, MI), and for each mouse the mean of three independent values was calculated.

**Statistical analysis**
Results were analyzed using the Student t test, Mann–Whitney U test, or log-rank test.

**RESULTS AND DISCUSSION**
CamK-HA mice express HA specifically in CNS neurons
To identify the parameters governing the vulnerability of CNS neurons to an attack by self-reactive CD8<sup>+</sup> T cells, we generated a mouse model, called CamK-HA, in which the HA of influenza virus is expressed in CNS neurons. These mice were obtained by crossing the CamK-iCre BAC transgenic mice (7) with the Rosa26<sup>tm(HA)1Lib</sup> mice (8) (Supplemental Fig. 2A). As expected, Cre-mediated genomic DNA recombination and
CD8 T cells induce central diabetes insipidus in CamK-HA mice

To analyze whether effector CD8 T cells can mediate neuronal cell death, we generated CTLs from CL4-TCR mice in vitro that produce large amounts of granzyme B (GrB), TNF-α, and IFN-γ (Supplemental Fig. 1B). Transfer of 3 × 10⁷ of HA-specific CTLs had dramatic effects on all CamK-HA mice, whereas the littermate control mice were totally unaffected (Fig. 1). CamK-HA mice underwent weight loss (Fig. 1A) and developed clear neurologic signs associating trembling and hypoaactivity, without obvious paralysis. A profound defect in motor performance, as assessed by the Rota-Rod test, was present during this acute phase (Fig. 1B). Overall, 43% of CamK-HA mice (13 of 30) died, mostly within 14 d posttransfer (Fig. 1C). Immunohistochemical analysis of the CNS 7 d after transfer revealed a prominent inflammation and T cell infiltration in the spinal cord and brain, predominantly in the hypothalamus, brain stem, and medulla oblongata of CamK-HA mice (Fig. 1D), whereas no infiltration was detected in control mice. During this acute phase, 90.5% of the CNS-infiltrating T cells were CD4⁺ CD8⁺ T cells, the vast majority being of donor origin as assessed by expression of the transgenic TCRβ-chain and of GFP (Fig. 1E). On average, 9.5% of the infiltrating T cells were endogenous CD4⁺ T cells (Fig. 1E). After this acute encephalomyelitis, the surviving CamK-HA recipient mice fully recovered their initial weight, behavior and Rota-Rod performances (Fig. 1B). Transfer of polyclonal (non-HA-specific) CTLs in CamK-HA (n = 6) and control (n = 6) mice did not promote any neurologic manifestation as assessed clinically or histologically (data not shown), underlining the strict Ag dependency of the phenotype observed after transfer of HA-specific CTLs in CamK-HA recipients.

Unexpectedly, however, from day 10 after CTL transfer, all surviving CamK-HA recipients (n = 17) developed severe polyuria without glycosuria. The urine volume was on average 11 times higher in CamK-HA mice (2.57 ± 0.39 ml/12 h) compared with that in controls (0.23 ± 0.06 ml/12 h) accompanied by increased water consumption (4.59 ± 0.44 ml/12 h versus 0.59 ± 0.22 ml/12 h in controls) (Fig. 2A). The clinical signs observed in chronically affected animals were

the resulting transcription of HA occurred only in CamK-HA mice and were confined to the brain and spinal cord (Supplemental Fig. 2B, 2C). Immunofluorescence performed on brain sections showed HA⁺ neurons only in CamK-HA mice (Supplemental Fig. 2E, 2F). This expression of HA specifically in CNS neurons did not lead to any spontaneous immunological or neurologic phenotype.

FIGURE 2. CamK-HA mice develop CD8 T cell-mediated central diabetes insipidus. (A) Urine volume and water consumption in CamK-HA (n = 8) and control (n = 7) mice after injection of HA-specific CTLs. Horizontal bars indicate means. p = 0.0003 (Mann–Whitney U test). (B) Urine volume of CamK-HA mice (n = 8) before and after injection of a vasopressin analogue. p = 0.0003 (paired Student t test). (C) Plasma vasopressin levels of CamK-HA (n = 7) and control (n = 8) recipients. p = 0.0012 (Mann–Whitney U test). (A–C) All measurements were performed in metabolic cages, at least 20 d after T cell transfer. (D) AVP staining (red) in the supraoptic nucleus 56 d after T cell transfer in CamK-HA and control mice. OC, optic chiasma. Scale bar, 100 μm. (E) Anti-CD3 staining of CamK-HA recipients in supraoptic and paraventricular nuclei at day 4 after injection of HA-specific CTLs. Blue, hematoxylin; brown, CD3. Similar results were obtained in three mice. Scale bars, 100 μm. (F) AVP and GrB staining 4 d after transfer of HA-specific CTLs showing GrB⁺ T cells (arrowheads) in close contact with AVP-expressing neurons in CamK-HA mice. Some cells showed GrB⁺ granules polarized against AVP-expressing neurons (enlarged box). Red, GrB; blue, AVP. Similar results were obtained in five mice. Scale bar, 5 μm. (G) Apoptotic bodies (arrows) and condensed nuclei in AVP⁺ neurons in the paraventricular nucleus of CamK-HA mice 6 d after T cell transfer. Brown, AVP; blue, hematoxylin. Similar results were obtained in three mice per group. Scale bars, 10 μm. (H) Activated caspase 3 staining revealing positive neurons in the paraventricular nucleus of a CamK-HA mouse 6 d after CTL transfer. The inset shows a caspase 3⁺ cell with neuronal morphology and condensed nucleus (arrow). Blue, hematoxylin; brown, caspase 3. Scale bar, 20 μm.
In contrast, GFP+ T cells were detected in the CNS of CCL2 (MCP-1) and CXCL12 (SDF-1), which are constitutively independent attraction by locally released chemokines such as of the time points explored (Fig. 3A), ruling out their Ag-dependent recruitment of CTLs to the hypothalamus or to higher expression of HA and/or MHC class I molecules locally. To investigate the first possibility, we transferred GFP-transduced IFN-γ-dependent HA-specific CTLs, polyuria was corrected by injection of the AVP analogue desmopressin (Fig. 2B), demonstrating normal kidney responsiveness to AVP. Moreover, AVP concentrations were profoundly decreased in the serum of CamK-HA recipients (Fig. 2C), indicating deficient secretion of AVP by the hypothalamo-neurohypophysis axis. Histological analysis of the hypothalamus revealed a dramatic loss of AVP-expressing magnocellular neurons in CamK-HA recipients (Fig. 2D). Collectively, these data clearly demonstrate the central origin of diabetes insipidus in our model.

Early after CD8 T cell transfer, we observed that T cells accumulated in the hypothalamus, notably in the supraoptic and paraventricular nuclei (Fig. 2E). The GrB+ T cells were often found in close apposition to AVP-expressing neurons (Fig. 2F), with some in which the GrB+ granules faced AVP+ neurons, suggesting polarized degranulation (Fig. 2F). In some of the AVP-expressing neurons, signs of apoptosis such as apoptotic bodies and condensed nuclei were clearly present (Fig. 2G). Some hypothalamic cells with neuronal morphology also expressed the activated form of caspase 3 (Fig. 2H). These data strongly suggest that the transferred CTLs perform GrB-mediated killing of Ag-expressing AVP+ neurons.

**Mechanism of the preferential targeting of vasopressin-producing neurons by CD8 T cells**

Surprisingly, although HA was widely expressed in CNS neurons of CamK-HA mice, CD8 T cell-mediated permanent damage was focal. This apparent paradox could be due to Ag-independent recruitment of CTLs to the hypothalamus or to higher expression of HA and/or MHC class I molecules locally. To investigate the first possibility, we transfected GFP-expressing HA-specific CTLs and followed the kinetics of CD8 T cell migration into the CNS. We did not detect any CD8 T cells in the hypothalamus of littermate control mice at any of the time points explored (Fig. 3A), ruling out their Ag-independent attraction by locally released chemokines such as CCL2 (MCP-1) and CXCL12 (SDF-1), which are constitutively expressed by AVP-producing hypothalamic neurons (10). In contrast, GFP+ T cells were detected in the CNS of CamK-HA recipients as early as 24 h after transfer with a marked accumulation of CTLs in the hypothalamus at 72 h (Fig. 3A), whereas the number of T cells in the rest of the brain was unchanged.

Second, since the CTL infiltration and subsequent destruction of hypothalamic magnocellular neurons depended upon the presence of the HA Ag, we compared its expression levels in different regions of the brain of CamK-HA mice. Using quantitative RT-PCR, comparable HA levels were found in the cerebellum, cortex, hippocampus, and supraoptic nucleus (Supplemental Fig. 2D).

**FIGURE 3.** Upregulation of MHC class I molecules and loss of AVP+ neurons after T cell transfer in CamK-HA mice are IFN-γ dependent. CamK-HA and control mice were examined 24, 48, and 72 h after transfer of GFP+ HA-specific CD8 T cells for T cell infiltration (A) and MHC class I expression (B). Similar results were obtained in three mice per group. Green, GFP+ CD8 T cells; red, H-2Kd. Scale bars, 25 μm. (C) Maximum weight loss after transfer of HA-specific IFN-γ−/− CD8 T cells as percent of initial weight (CamK-HA, n = 12; controls, n = 5). (D) Characterization of CNS-infiltrating cells in CamK-HA recipients 6–8 d after transfer of GFP-transduced IFN-γ−/− CD8 T cell transfer. Upper left panel, T cells detected as CD45+ Thy1.2+ cells (blue gate). Upper right panel, CD4 expression among CNS-infiltrating T cells. Lower left panel, GFP expression in CD8+ CD4+ Thy1.2+ cells (blue curve) compared with that in CD4+ Thy1.2+ cells (red curve). Lower right panel, Vβ8 expression among CD8+ CD4+ Thy1.2+ cells (blue curve) compared with that among CD4+ Thy1.2+ cells (red curve). Similar results were obtained in five CamK-HA mice. (E) Analysis of MHC class I and AVP expression in the supraoptic nucleus of CamK-HA and control mice 5 d after transfer of GFP+ IFN-γ−/− CTLs. Similar results were obtained in three mice per group. Green, GFP+ IFN-γ−/− CTLs (left); red, H-2Kd (middle) and AVP (right). Scale bar, 25 μm. OC, optic chiasma.
Finally, we investigated whether the accumulation of CD8 T cells in the hypothalamus correlated with the expression of MHC class I molecules. The expression of Kd molecules was barely detectable in CamK-HA and control mice, either unmanipulated or 24 h after CTL transfer. Importantly, MHC class I expression was strongly upregulated at 48 h in the supraoptic nucleus of all CamK-HA mice but not in control mice (Fig. 3B). A very tight correlation existed between T cell infiltration, MHC class I upregulation, and loss of AVP-producing neurons. These data therefore strongly suggest that an Ag-dependent mechanism promotes CD8 T cell infiltration in the CNS and that preferential upregulation of MHC class I in the supraoptic and paraventricular nuclei favors accumulation of CTLs and targeting of the magnocellular neurons. Consistent with this hypothesis, these hypophysal lamic neurons have been shown to strongly upregulate MHC class I molecules in rats (11).

To test if MHC class I upregulation and hypophysal lamic neuron destruction are dependent on IFN-γ production by the transferred CD8 T cells, we injected CamK-HA mice with HA-specific CTLs from IFN-γ−/− CLA4-TCR mice (Supplemental Fig. 1B). Transfer of 3 × 10^7 of such CTLs induced transient weight loss in 75% of the recipients (9 of 12 mice), albeit not as marked as with IFN-γ-expressing CTLs (Fig. 3C). All mice survived but none subsequently exhibited signs of diabetes insipidus. On average, 80.4% of the CNS-infiltrating T cells were CD8^+ CD4^− T cells, 86% of them expressing the transgenic TCRβ-chain (Fig. 3D). Histological analysis 5 d after transfer of IFN-γ−/− CTLs revealed moderate infiltration of T cells in the hypothalamus, but there was hardly any MHC class I upregulation, and AVP expression remained normal in these mice (Fig. 3E). These data highlight the preferential vulnerability of the AVP-producing neurons to CD8 T cells owing to their propensity to upregulate MHC class I molecules in response to inflammation.

In humans, 30–50% of central diabetes insipidus cases are considered idiopathic (9). Strong but indirect evidence gives credence to the autoimmune nature of at least some of these cases. First, pituitary stalk biopsies performed at the onset of diabetes insipidus show T cell-rich inflammatory infiltrates. One published report illustrates a predominance of CD8 T cells among the invading T cells (12), and we have made similar observations in the biopsy material from another patient with recent-onset central diabetes insipidus (data not shown). Notably, in our mouse model, loss of hypophysal lamic magnocellular neurons is found in the few autopsied cases (13). In addition, autoantibodies to AVP^+ neurons are frequently detected in idiopathic cases (14). Importantly, central diabetes insipidus is often associated with other autoimmune diseases and, in particular, can be a manifestation of the autoimmune polyendocrine syndrome caused by AIRE mutations (15). Finally, lymphocytic hypophysitis as well as central diabetes insipidus have recently been added to the list of immune-related adverse events of anti-CTLA-4 therapy for neoplastic diseases (16). Our data reinforce the concept that some, if not most, cases of idiopathic central diabetes insipidus could be caused by an autoimmune reaction against hypophysal lamic neuronal Ags mediated by CD8^+ T cells.

Although the clinical manifestations of diabetes insipidus are satisfactorily controlled by substitution therapy, the animal model described in this study provides definitive evidence for an immune-mediated origin and new mechanistic information regarding the pathogenesis of this CNS autoimmune disease. We also provide definitive evidence supporting the concept that autoimmune diseases targeting CNS neurons can be mediated by CD8 T cells, thereby pointing at new therapeutic targets.

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Disclosures

The authors have no financial conflicts of interest.

References


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Supplemental Fig. 1. Phenotype of the transferred CTL. (A) Before transfer, the purity of the CTL population was assessed by FACS following staining for CD8 and Vβ8 (right panel) or unstained (left panel). The data are representative of more than 10 independent HA-specific CTL cultures and 3 HA-specific IFN-γ−/− CTL cultures. (B) HA-specific CTL (top) and HA-specific IFN-γ−/− CTL (bottom) were stimulated with PMA (1 μg/ml) and ionomycin (1 μg/ml) for 4 h and stained using granzyme B-, TNF-α- and IFN-γ-specific antibodies (blue curves) or with isotype controls (red curves).
Supplemental Fig. 2. DNA recombination and HA expression in CamK-HA mice. (A) Transgenic loci of Rosa26<sup>tm(HA)1Lib</sup> (top), CamK-iCre (middle), and CamK-HA (bottom) mice. SA=splice acceptor sequence, tpA=transcriptional stop sequence, HA=hemagglutinin, P1 to P4=primers, ▶️=loxP site. (B) Cre-mediated recombination was assessed by PCR on genomic DNA extracted from different organs of CamK-HA and Rosa26<sup>tm(HA)1Lib</sup> mice using the primers P1 and P2 (620 bp). Similar results were obtained in three mice per group. (C) Transcription of HA in different tissues from CamK-HA and Rosa26<sup>tm(HA)1Lib</sup> mice was assessed by nested RT-PCR using the primers P1 and P2 for the first PCR and P3 and P4 for the second PCR (336 bp). Similar results were obtained in three mice per group. Tissue abbreviations: B, brain; Sc,
spinal cord; T, thymus; Sp, spleen; K, kidney; M, muscle; H, heart; Li, liver; Lu, lung; I, intestine; LN, pooled axillary, brachial and inguinal lymph nodes. (D) HA expression in different brain regions of CamK-HA mice. Quantitative RT-PCR analysis showing a comparable level of HA expression in cerebellum (Cb), cortex (Co), hippocampus (Hip) and supraoptic nucleus (SON) of CamK-HA mice. HA transcripts were normalized to HPRT transcripts and mRNA levels are shown relative to the mean of the expression level in the cerebellum, which was set to 1. Cerebellum n=3, cortex and hippocampus n=4, pooled dissected supraoptic nucleus from 3 mice. Immunohistofluorescence showing HA protein expression in cerebellar Purkinje neurons (E) and cortical neurons (F) of CamK-HA mice. n=3, green: HA. (E) Red: calbindin-28K (Calb), scale bar=50 μm. (F) Red: NeuN, scale bar=10 μm.