Molecular Mimicry between Neurons and an Intracerebral Pathogen Induces a CD8 T Cell-Mediated Autoimmune Disease

Monica Sanchez-Ruiz, Laura Wilden, Werner Müller, Werner Stenzel, Anna Brunn, Hrvoje Miletic, Dirk Schlüter and Martina Deckert

_J Immunol_ 2008; 180:8421-8433; ;
doi: 10.4049/jimmunol.180.12.8421
http://www.jimmunol.org/content/180/12/8421

References
This article cites 36 articles, 19 of which you can access for free at: http://www.jimmunol.org/content/180/12/8421.full#ref-list-1

Subscription
Information about subscribing to _The Journal of Immunology_ is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Molecular Mimicry between Neurons and an Intracerebral Pathogen Induces a CD8 T Cell-Mediated Autoimmune Disease

Monica Sanchez-Ruiz,* Laura Wilden,* Werner Müller,†§ Werner Stenzel,* Anna Brunn,* Hrvoje Miletic,* Dirk Schlüter,‡‡ and Martina Deckert*‡

To identify basic mechanisms of how infections may induce a neuron-specific autoimmune response, we generated mice expressing OVA as neuronal autoantigen under control of the neuron-specific enolase promoter (NSE-OVA mice). Intracerebral, but not systemic, infection with attenuated Listeria monocytogenes-secreting OVA induced an atactic-paretic neurological syndrome in NSE-OVA mice after bacterial clearance from the brain, whereas wild-type mice remained healthy. Immunization with attenuated Listeria monocytogenes-secreting OVA before intracerebral infection strongly increased the number of intracerebral OVA-specific CD8 T cells aggravating neurological disease. T cell depletion and adoptive transfer experiments identified CD8 T cells as decisive mediators of the autoimmune disease. Importantly, NSE-OVA mice having received OVA-specific TCR transgenic CD8 T cells developed an accelerated, more severe, and extended neurological disease. Adoptively transferred pathogenic CD8 T cells specifically homed to OVA-expressing MHC class I neurons and, corresponding to the clinical symptoms, ∼30% of neurons in the anterior horn of the spinal cord became apoptotic. Thus, molecular mimicry between a pathogen and neurons can induce a CD8 T cell-mediated neurological disease, with its severity being influenced by the frequency of specific CD8 T cells, and its induction, but not its symptomatic phase, requiring the intracerebral presence of the pathogen. The Journal of Immunology, 2008, 180: 8421–8433.

With respect to the pathogenesis of various cerebral autoimmune diseases, an underlying infectious agent has been proposed. In fact, infections often precede cerebral autoimmune attacks, although a specific pathogen responsible for the induction of autoimmune CNS disorders has not been identified. Two major mutually not exclusive concepts have been proposed to explain how infections could induce autoimmune CNS diseases: 1) molecular mimicry, that is, the activation of autoimmune T cells, which are cross-reactive between a pathogen and self-Ags; and 2) bystander activation, that is, the activation of autoreactive T cells by nonspecific inflammatory events during an infection (1).

In clinically important autoimmune diseases of the CNS either neurons or oligodendrocytes serve as target cells of the autoimmune attack. Because oligodendrocytes and neurons can express MHC class I, but not MHC class II, Ags (2), CD8 T cells in contrast to CD4 T cells may directly destroy these cell types. In most autoimmune CNS disorders, CD8 T cells numerically dominate CD4 T cells (3). Surprisingly, the role of CD8 T cells has mainly been claimed to be regulatory, while CD4 T cells were regarded as major pathogenic players (4). However, there is direct evidence that in paraneoplastic syndromes (5) and Rasmussen’s encephalitis (6), CD8 T cells destroy neurons in a granzyme B-dependent mechanism, and myelin-specific CD8 T cells can induce severe CNS autoimmunity in mice with distinct clinical symptoms and pathology (7–9). Additionally, feline immunodeficiency virus-infected CD8 T cells can destroy dorsal root ganglion neurons in a CD154/CD40-dependent pathway (10).

So far, experimental models have failed to induce a CD8 T cell-mediated, clinically relevant autoimmune disease of neurons by an infection. Therefore, it is at present unclear under which circumstances a clinically relevant autoimmune response of pathogen-specific CD8 T cells against neurons can be induced. Because pathogen-specific T cells are primed in peripheral lymphoid organs, and even in exclusively extracerebral systemic infections, the CNS is immunosurveillance by pathogen-specific activated CD4 and CD8 T cells (11), and strictly peripheral infections may induce an autoimmune attack by cross-reactive T cells against neurons. However, the induction of an autoimmune response against neurons might also require a direct infection of the CNS that induces a local proinflammatory milieu and results in a further increased recruitment of pathogen-specific T cells.

To systematically analyze under which circumstances a clinically overt autoimmune attack against neurons can be induced by molecular mimicry between neurons and a pathogen, we have generated a murine model in which neurons express OVA, which has defined CD8 T cell epitopes, and infected these mice systemically or intracerebrally (i.c.) with Listeria monocytogenes (LM) secreting OVA or not. The model of listeriosis and the OVA Ag were chosen because 1) LM is a facultative intracellular bacterium, which can cause systemic and severe cerebral infections in humans and mice (12); 2) immunity against LM is primarily mediated by

*Abteilung für Neuropathologie, Universitätsklinikum Köln, Köln; †Helmholtz Zentrum für Infektionsforschung, Experimentelle Immunologie, Braunschweig; ‡Institut für Medizinische Mikrobiologie, Otto-von-Guericke Universität Magdeburg, Magdeburg, Germany; and ‡‡University of Manchester, Manchester, United Kingdom

Received for publication June 4, 2007. Accepted for publication April 14, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the Deutsche Forschungsgemeinschaft (Grant DE485/8-2) and the Zentrum für Molekulare Medizin Universität zu Köln (TV71).
2 Address correspondence and reprint requests to Dr. Martina Deckert, Department of Neuropathology, University Hospital of Cologne, Kerpener Strasse 62, 50924 Köln, Germany. E-mail address: neuropath@uni-koeln.de or Dr. Dirk Schlüter, Institut für Medizinische Mikrobiologie, Otto-von-Guericke Universität Magdeburg, Leipziger Strasse 44, 39120 Magdeburg, Germany. E-mail address: dirk.schlueter@medizin.uni-magdeburg.de

3 Abbreviations used in this paper: i.c., intracerebral; ΔactA, LMova, OVA-transgenic Listeria monocytogenes deleted of the actA gene; CNPase, 2’,3’-cyclic nucleotide 3’-phosphodiesterase; GFAP, glial fibrillary acidic protein; LM, Listeria monocytogenes; LMova, Listeria monocytogenes-secreting OVA; LLO, listeriolysin O; NeuN, neuronal nuclei; NSE, neuron-specific enolase; p.i., postinfection; TIR, transferrin receptor; WT, wild type.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00
CD8 T cells; 3) transgenic LM-secreting OVA (LMova) induces a strong CD8 T cell response against MHC class I-K\(^{b}\)-restricted OVA\(_{257-264}\); 4) OVA-transgenic LM deleted of the actA gene (\(\Delta\)actA LMova), which results in deficient direct cell-to-cell spread of the bacterium (13), induces a strong OVA\(_{257-264}\)-specific CD8 T cell response, thereby allowing the precise characterization and tracking of the ensuing immune response; and 5) both \(\Delta\)actA LM and \(\Delta\)actA LMova cause a mild, transient cerebral infection after i.c. inoculation in mice (14, 15).

Using this experimental system, we demonstrate for the first time that molecular mimicry between a pathogen and neurons can induce a CD8 T cell-mediated neurological disease, that the severity and duration of the disease were influenced by the frequency of i.c. OVA\(_{257-264}\)-specific CD8 T cells, and that induction of the disease required the presence of the pathogen in the CNS.

**Materials and Methods**

**Mice**

C57BL/6 mice (Charles River Laboratories), OT-I (C57BL/6-TgTcrTcrb1106Mjb/J, The Jackson Laboratory) mice carrying a transgenic TCR recognizing OVA\(_{257-264}\) in the context of H-2\(^{K}\), and B6.SIL-Ptprc\(^{+/−}\)Pepcd\(^{+/−}\)BoyJ mice (Charles River Laboratories), OT-I (C57BL/6-BoyJ, The Jackson Laboratory) were used.

**Isolation of splenocytes and cerebral leukocytes and flow cytometry analysis**

Animals were anesthetized with Metofane (Janssen Pharmaceutica) and intracardially perfused with PBS. Thereafter, cerebral and splenic leukocytes were isolated as described before (19). Isolated cerebral leukocytes were analyzed by double or triple immunofluorescence staining followed by flow cytometry as described (14). At each time point, brains or spleens of four mice were pooled.

**Isolation of splenocytes and cerebral leukocytes and flow cytometry analysis**

Animals were anesthetized with Metofane (Janssen Pharmaceutica) and intracardially perfused with PBS. Thereafter, cerebral and splenic leukocytes were isolated as described before (19). Isolated cerebral leukocytes were analyzed by double or triple immunofluorescence staining followed by flow cytometry as described (14). At each time point, brains or spleens of four mice were pooled.

**T cell depletion experiments**

For depletion of CD4 and/or CD8 T cells, mice were treated with either rat anti-mouse CD4 (clone GK1.5) and/or rat anti-mouse CD8 (clone PC61) Abs as described before (14), starting at day 12 of immunization (i.e., 3 days before i.c. infection). At the first 3 days of i.p. treatment, Abs (0.5 mg/mouse) were injected daily; thereafter, Abs were injected every third day. Control mice were treated with rat IgG (Sigma-Aldrich). Three days after i.c. infection, mice received ampicillin per os for 8 days. Efficacy of CD4 and CD8 T cell depletion was \(>95\%\) as controlled by flow cytometry.

**Adoptive cell transfer**

Leukocytes were isolated from the spleen of CD45.1 OT-I mice. CD8 T cells were purified from CD45.1 OT-I mice with the Midi-MACS system (Miltenyi Biotec) using CD8 T cell isolation kits. Purified CD8 T cells (2 \(\times\) 10\(^6\)) were injected i.v. in a volume of 200 \(\mu\)l 0.1 M PBS into naive recipient mice 1 day before infection. The purity of the isolated T cell population was \(>90\%\) as controlled by flow cytometry.

**Intracellular IFN-\(\gamma\) staining**

First, cerebral and splenic leukocytes were incubated with anti-CD16/32 followed by extracellular staining with anti-CD4-FITC and anti-CD8-PE, respectively, and anti-CD45-CyChrome (BD Biosciences). Leukocytes isolated from mice having received CD45.1\(^{+}\) OT-I CD8 T cells were stained with CD8-FITC in combination with CD45.1-CyChrome and CD45.2-CyChrome, respectively. Thereafter, cells were washed twice in 0.1 M PBS, fixed with Cytofix/Cytoperm solution (BD Biosciences), and stained intracellularly with anti-IFN-\(\gamma\)-PE (clone XMG1.2, BD Biosciences). The final washing step was performed with BD **Materials and Methods**

**Mice**

C57BL/6 mice (Charles River Laboratories), OT-I (C57BL/6-TgTcrTcrb1106Mjb/J, The Jackson Laboratory) mice carrying a transgenic TCR recognizing OVA\(_{257-264}\) in the context of H-2\(^{K}\), and B6.SIL-Ptprc\(^{+/−}\)Pepcd\(^{+/−}\)BoyJ mice (Charles River Laboratories), OT-I (C57BL/6-BoyJ, The Jackson Laboratory) were used.

**Materials and Methods**

**Mice**

C57BL/6 mice (Charles River Laboratories), OT-I (C57BL/6-TgTcrTcrb1106Mjb/J, The Jackson Laboratory) mice carrying a transgenic TCR recognizing OVA\(_{257-264}\) in the context of H-2\(^{K}\), and B6.SIL-Ptprc\(^{+/−}\)Pepcd\(^{+/−}\)BoyJ mice (Charles River Laboratories), OT-I (C57BL/6-BoyJ, The Jackson Laboratory) were used.

**Materials and Methods**

**Mice**

C57BL/6 mice (Charles River Laboratories), OT-I (C57BL/6-TgTcrTcrb1106Mjb/J, The Jackson Laboratory) mice carrying a transgenic TCR recognizing OVA\(_{257-264}\) in the context of H-2\(^{K}\), and B6.SIL-Ptprc\(^{+/−}\)Pepcd\(^{+/−}\)BoyJ mice (Charles River Laboratories), OT-I (C57BL/6-BoyJ, The Jackson Laboratory) were used.

**Materials and Methods**

**Mice**

C57BL/6 mice (Charles River Laboratories), OT-I (C57BL/6-TgTcrTcrb1106Mjb/J, The Jackson Laboratory) mice carrying a transgenic TCR recognizing OVA\(_{257-264}\) in the context of H-2\(^{K}\), and B6.SIL-Ptprc\(^{+/−}\)Pepcd\(^{+/−}\)BoyJ mice (Charles River Laboratories), OT-I (C57BL/6-BoyJ, The Jackson Laboratory) were used.

**Materials and Methods**

**Mice**

C57BL/6 mice (Charles River Laboratories), OT-I (C57BL/6-TgTcrTcrb1106Mjb/J, The Jackson Laboratory) mice carrying a transgenic TCR recognizing OVA\(_{257-264}\) in the context of H-2\(^{K}\), and B6.SIL-Ptprc\(^{+/−}\)Pepcd\(^{+/−}\)BoyJ mice (Charles River Laboratories), OT-I (C57BL/6-BoyJ, The Jackson Laboratory) were used.

**Materials and Methods**

**Mice**

C57BL/6 mice (Charles River Laboratories), OT-I (C57BL/6-TgTcrTcrb1106Mjb/J, The Jackson Laboratory) mice carrying a transgenic TCR recognizing OVA\(_{257-264}\) in the context of H-2\(^{K}\), and B6.SIL-Ptprc\(^{+/−}\)Pepcd\(^{+/−}\)BoyJ mice (Charles River Laboratories), OT-I (C57BL/6-BoyJ, The Jackson Laboratory) were used.

**Materials and Methods**

**Mice**

C57BL/6 mice (Charles River Laboratories), OT-I (C57BL/6-TgTcrTcrb1106Mjb/J, The Jackson Laboratory) mice carrying a transgenic TCR recognizing OVA\(_{257-264}\) in the context of H-2\(^{K}\), and B6.SIL-Ptprc\(^{+/−}\)Pepcd\(^{+/−}\)BoyJ mice (Charles River Laboratories), OT-I (C57BL/6-BoyJ, The Jackson Laboratory) were used.
of NSE-OVA transgenic mice. A, Southern blot analysis of tail DNA. Founders were analyzed for presence of the transgene. Lanes 1–5 correspond to five positive transgenic mice, lane 6 corresponds to a negative control C57BL/6 WT mouse, and lane 7 corresponds to the pNSE-OVA plasmid as positive control. B, OVA protein is expressed in brain (lane 1), but is absent from thymus (lane 2), lung (lane 3), liver (lane 4), spleen (lane 5), kidney (lane 6), and heart (lane 7) of NSE-OVA mice as well as from the brain of a WT mouse (lane 8). Western blot analysis with rabbit anti-OVA. C–F, In an NSE-OVA mouse, OVA is specifically and exclusively expressed on the cell membrane of neurons in the frontal cortex (C), but is consistently absent from GFAP-expressing astrocytes (D), CNPase-expressing oligodendrocytes (E), and F4/80-expressing macrophages/microglia (F). Double immunofluorescence staining: OVA is shown in red (C–F).

Permeabilization was performed with 0.1% BSA in 0.1% PBS. To analyze IFN-γ production of Listeria-specific CD4 and CD8 T cells from mice infected i.c. with ΔactA LMova, isolated leukocytes were stimulated with OVA257–264 (CD8 specific, 10−8 M) or listeriolysin O (LLO),190–201 (CD4 specific, 10−8 M) peptide in the presence of GolgiPlug (1 μl/ml, Cytofix/Cytoperm kit, BD Biosciences) at 37°C for 4 h before IFN-γ staining and flow cytometric analysis.

**Statistical evaluation**

Differences in the i.c. bacterial load between the various experimental groups were evaluated using a two-tailed Student’s t test. In NSE-OVA and C57BL/6 mice having been adoptively transferred with OT-I cells followed by an i.c. infection with ΔactA LMova, the numbers of viable and apoptotic neurons were counted in high-power fields of cresyl violet-stained spinal cord paraffin sections of at least two animals per experimental group. Differences were determined using the Mann-Whitney U test. To test for the clinical score, a two-tailed Mann-Whitney U test was applied. Data are presented as means ± SD; p < 0.05 was considered significant. Experiments were performed at least in duplicate.

**Results**

**Generation and characterization of NSE-OVA transgenic mice**

To direct OVA expression to the cell surface of CNS neurons, a NSE-TIR/OVA construct was generated as described in Materials and Methods. The TIR/OVA fragment was isolated, microinjected into the pronuclei of C57BL/6 fertilized eggs, and the presence of the OVA transgene was confirmed in mutant mice (Fig. 1A). The TIR-OVA fusion protein (45 kDa) was expressed CNS specifically and was absent from thymus, heart, lung, liver, spleen, and kidney of NSE-OVA mice (Fig. 1B).

OVA was ubiquitously expressed on neurons of the CNS, including cortex, basal ganglia, hippocampus, cerebellum, brain stem, and spinal cord (Fig. 1C). GFAP+ astrocytes, CNPase+ oligodendrocytes, as well as F4/80+ macrophages/microglia were consistently OVA negative (Fig. 1D–F). In contrast to NSE-OVA mice, normal wild-type (WT) mice did not express OVA in any cell of the CNS (data not shown).

NSE-OVA mice were observed for up to 1 year and none of these animals developed any clinical symptoms spontaneously. A detailed histopathological investigation of the brain and spinal cord of NSE-OVA mice up to the age of 10 mo did not reveal any abnormalities, illustrating that OVA expression of neurons does not cause disease (data not shown).

A systemic infection with OVA-transgenic LM does not induce a cerebral autoimmune disease in NSE-OVA mice

We have previously shown that after i.p. infection with ΔactA LM, LM-specific CD8 T cells are recruited to the CNS in the absence of a cerebral invasion by LM (11). To analyze whether such an immunosurveillance of the CNS by pathogen-specific CD8 T cells might cause disease in animals expressing cross-reactive CD8 T cell epitopes on neurons, we infected NSE-OVA and C57BL/6 WT mice i.p. with either ΔactA LM or ΔactA LMova.

Systemic infection of both NSE-OVA and WT mice with ΔactA LMova induced splenic OVA257–264-specific CD8 T cells in both strains of mice without significant differences (Fig. 2A). Additionally, i.p. infection with ΔactA LMova also resulted in the recruitment of a similar, small number of OVA257–264-specific CD8 T cells to the CNS of both WT and NSE-OVA mice (Fig. 2B). In agreement with previously published data (11), immunohistochemistry revealed that CD8 T cells were located in the leptomeninges, perivascularly, and occasionally in the brain parenchyma (data not shown). In contrast, after infection with OVA-negative ΔactA LM, OVA257–264-specific CD8 T cells were not detected in spleen or in brain (data not shown).

In agreement with data published previously (11), the determination of CFU in the brain at various time points after i.p. infection (days 1–7) confirmed that LM did not invade the CNS in any of the experimental groups (data not shown).

Importantly, mice of all experimental groups remained healthy and had a clinical score of 0 on a scale that clinically evaluated neurological symptoms. Additionally, a detailed histopathological analysis showed that neurons in the brain and spinal cord did not show any abnormalities and were not associated with any inflammatory cells (data not shown).
These findings illustrate that NSE-OVA mice developed an OVA-specific CD8 T cell response after i.p. infection with ΔactA LMova and were not tolerant against OVA. Additionally, the recruitment of pathogen-specific (OVA257-264) CD8 T cells to the CNS was not sufficient to induce a neurological disease in mice expressing OVA on neurons.

**Cerebral infection with OVA-transgenic LM induces a sustained neurological disorder in NSE-OVA mice**

To analyze whether a direct infection of the CNS by LM expressing OVA alters the course of disease in NSE-OVA mice as compared with i.p. infection, NSE-OVA and WT mice were infected i.c. with ΔactA LMova or ΔactA LM.

In agreement with previous results (14), in both NSE-OVA and WT mice, i.e. application of OVA-negative ΔactA LM resulted in a clinically asymptomatic cerebral listeriosis (score of 0 in both groups). Histopathology showed that both groups of mice developed a mild meningoencephalitis, with LM being restricted to the leptomeninges, the choroid plexus, and the ependyma of the lateral ventricle. Importantly, there was only a marginal infiltration of the brain parenchyma by CD4 and CD8 T cells, which were scattered diffusely throughout the brain, without any pathology of neurons in both groups of mice as demonstrated by immunohistochemistry on representative serial sections throughout the brain (data not shown). Thus, expression of OVA on neurons does not alter the mild course of cerebral listeriosis after i.c. application of attenuated ΔactA LM, which extends and confirms previous data (14).

In contrast, i.c. infection of NSE-OVA mice with OVA-secreting ΔactA LM induced a neurological disease characterized by irritability and tremor, coordination disturbance, abnormal gait, hind limb weakness, and in some mice by an additional spontaneous slight hind limb ataxia (Fig. 3A). Clinical symptoms started in individual NSE-OVA mice at day 3 p.i., were manifest in 100% of mice at day 5 p.i., reached a maximum at day 11 p.i. (score of 3.5), declined thereafter, and were resolved at day 23 p.i. (Fig. 3, A and

---

**FIGURE 2.** Numbers of OVA257-264-specific cells in brain and spleen of NSE-OVA and C57BL/6 WT mice. Numbers of splenic and cerebral CD8 T cells specific for OVA257-264 in NSE-OVA and WT mice were determined by IFN-γ ELISPOT at day 8 after i.p. application of ΔactA LMova. For the analysis, brains (B) and spleens (A) of four mice per experimental group were pooled at each time point p.i. Data represent the means ± SD of triplicate wells. One of two experiments, which yielded identical results, is shown.

---

**FIGURE 3.** Clinical score, disease incidence, and kinetics of bacterial replication and elimination in NSE-OVA and C57BL/6 WT mice following i.c. infection. A and B. Only NSE-OVA mice having been infected i.c. with ΔactA LMova develop clinically overt neurological disease. Five mice per group were analyzed. The difference in the clinical score between NSE-OVA and C57BL/6 mice was statistically significant (p < 0.05 at days 7, 9, 11, 14, and 16 p.i.). Experiments were performed in duplicate. One of two experiments, which yielded identical results, is shown. C and D. Upon i.c. infection with ΔactA LM (C) and ΔactA LMova (D), respectively, NSE-OVA and WT mice eliminate both strains of bacteria with identical efficiency. Data represent the means ± SD of three to four animals per time point and experimental group. Differences in the number of CFU between the two genotypes were statistically not significant (p > 0.05). One of two representative experiments, which gave identical results, is shown. *, p < 0.05; n.s., not significant.
B). Importantly, i.c. infection of WT mice with ∆actA LMova did not induce a clinically overt neurological disease, and WT mice always had a clinical score of 0 (Fig. 3, A and B).

To rule out that the development of disease in NSE-OVA mice infected with ∆actA LMova was caused by an insufficient control of LM in these animals, i.e. CFUs were determined. As illustrated in Fig. 3, C and D, CFUs of NSE-OVA and WT mice did not differ after infection with either ∆actA LM (Fig. 3C) of ∆actA LMova (Fig. 3D) and bacteria were eliminated with the same kinetics from the CNS. Thus, NSE-OVA mice infected i.c. with ∆actA LMova reached the maximum of disease activity, when the pathogen had already been eliminated from the CNS.

In conclusion, these experiments show that only an i.c. infection with ∆actA LMova induced a neurological disease in NSE-OVA transgenic mice, which was not caused by LM per se.

A systemic immunization with ∆actA LMova before i.c. infection with ∆actA LMova aggravates the neurological disease of NSE-OVA mice

In systemic and cerebral listeriosis, an immunization with viable LM before challenge infection results in a more rapid elimination of the pathogen, which is mediated by an increased frequency of LM-specific T cells, especially CD8 T cells (15). To analyze how a systemic immunization with ∆actA LMova affects the neurological disease of NSE-OVA mice before i.c. infection with the same bacterial strain, NSE-OVA and WT mice were immunized i.p. with 1 × 10⁶ ∆actA LMova and were i.c. challenged with 1 × 10³ ∆actA LMova 14 days thereafter.

As expected, immunized NSE-OVA and WT mice eliminated ∆actA LMova more rapidly from the CNS than did nonimmunized mice, and in both strains of immunized mice bacteria were not detectable beyond day 5 after i.c. infection (Fig. 4A).

Importantly, immunized NSE-OVA mice developed a neurological disease, which in individual animals started at day 3 after i.c. infection (Fig. 4, B and C). At day 5 p.i., all NSE-OVA mice were diseased, and clinical symptoms further progressed until day 11 p.i. with a mean score 5.0 (Fig. 4, B and C). Thereafter, disease severity declined and all mice recovered up to day 23 p.i. Thus, in immunized NSE-OVA mice the disease was even more severe than in nonimmunized NSE-OVA mice after i.c. infection with ∆actA LMova with respect to the clinical score (p < 0.05 at days 7, 9, and 11 p.i; see Fig. 3A vs Fig. 4B). In contrast, immunized WT mice did not develop a neurological disease after i.c. infection with ∆actA LMova (Fig. 4, B and C).

These experiments show that despite a more effective elimination of LM in immunized mice, the neurological disease is even more severe in immunized NSE-OVA mice.

Intracerebral CD4 and CD8 T cells including OVA₂₅⁷–₂₆₄-specific CD8 T cells increase in the brains of NSE-OVA mice having been infected i.c. with ∆actA LMova

To evaluate how the expression of OVA on neurons affects the local T cell response, the i.c. immune reaction of immunized and nonimmunized NSE-OVA and WT mice after i.c. infection with ∆actA LMova was studied.

After i.c. infection with ∆actA LMova the number of i.c. CD8 T cells increased in both strains of mice, with higher numbers found in immunized than nonimmunized mice (Fig. 5A). However, the kinetics of CD8 T cells was different in WT and NSE-OVA mice. Up to day 7 p.i., CD8 T cells increased in both nonimmunized and immunized NSE-OVA and WT mice. Whereas numbers of i.c. CD8 T cells gradually decreased in both nonimmunized and immunized WT animals thereafter, numbers of i.c. CD8 T cells further increased, especially in immunized mice, in parallel to the disease activity up to day 14 p.i. At this time point, numbers of i.c. CD8 T cells were increased in immunized NSE-OVA mice as compared with WT mice (Fig. 5A). Thereafter, numbers of CD8 T cells decreased in parallel to the regressing disease activity in NSE-OVA mice.

To prove that the increased number of CD8 T cells included OVA₂₅⁷–₂₆₄-specific CD8 T cells, the frequency of CD8 T cells specific for this epitope was determined in immunized animals. As illustrated in Fig. 5B, immunized NSE-OVA mice harbored elevated numbers of OVA₂₅⁷–₂₆₄-specific cells in the CNS as compared with WT animals at days 5, 8, and 15 p.i. However, in contrast to the total number of CD8 T cells, OVA₂₅⁷–₂₆₄-specific cells did not further increase but declined in NSE-OVA mice from day 5 to 15 p.i. Interestingly, in the spleen, the number of OVA₂₅⁷–₂₆₄-specific CD8 T cells did not differ between NSE-OVA and WT mice beyond day 5 p.i. (Fig. 5C).

Intracellular IFN-γ FACS staining further confirmed these ELISPOT data, showing exclusively IFN-γ-producing OVA₂₅⁷–₂₆₄-specific CD8 T cells in brain and spleen of ∆actA LMova-infected NSE-OVA and WT mice and an increased number of these cells in NSE-OVA mice as compared with WT mice at both days 8 and 15 p.i. (data not shown). Additionally, flow cytometry revealed...
that OVA-specific CD8 T cells accounted for 33.5, 67.1, and 53.7% of the whole i.c. CD8 T cell population in NSE-OVA mice at days 5, 8, and 15 p.i., respectively (Fig. 5D). In WT mice, the percentage of OVA-specific CD8 T cells among the bulk of i.c. CD8 T cells was slightly lower, and they accounted for 25.3, 52.7, and 41.8% of all i.c. CD8 T cells at days 5, 8, and 15 p.i. (Fig. 5D).

Because CD4 T cells might play an important regulatory role for the i.c. CD8 T cell response, we also determined the kinetics of i.c. CD4 T cells. The total number of i.c. CD4 T cells increased in nonimmunized and immunized mice were determined by flow cytometry. For FACS analysis of i.c. leukocytes, brains of four mice per experimental group were pooled at each time point after i.c. infection. Experiments were performed in duplicate. Data are expressed as numbers of cells of the respective cell type per brain. B, C, F, and G. The number of cerebral (B and F) and splenic (C and G) cells specific for OVA257-264 (B and C) and OVA123-139 or LLO190-201 (F and G) was determined by IFN-γ ELISPOT in NSE-OVA and WT mice at days 5, 8, and 15 after i.c. infection with ΔactA LMova. All animals received a primary i.p. infection 15 days before i.c. infection. D. The percentage of i.c. OVA257-264-specific CD8 T cells was determined by intracellular IFN-γ FACS staining in i.p. immunized NSE-OVA and WT mice at days 5, 8, and 15 after i.c. challenge infection with ΔactA LMova. After isolation of leukocytes from the brain, cells were restimulated with OVA257-264 peptide, and the percentage of IFN-γ-producing CD8 T cells among all CD8 T cells is shown. For the analyses described in A–G, brains and spleens of four mice were pooled. The means ± SD of triplicate wells are shown.
These ELISPOT data were confirmed by determination of the number of i.c. and splenic LLO\textsubscript{190–201}-specific CD4 T cells by IFN-\(\gamma\)/H9253 staining and flow cytometry (data not shown).

In conclusion, these experiments indicate that the elevated number of pathogen-specific, potentially cross-reactive CD8 T cells in the CNS of NSE-OVA mice is forced by a local, CNS-specific factor, presumably OVA expression of neurons.

Intracerebral CD8 T cells form neuron-associated infiltrates in the brains of NSE-OVA transgenic mice

Having established that the neurological disease of NSE-OVA mice is characterized by an increased number of CD8 T cells, including OVA-specific CD8 T cells in the CNS, we next determined the association of T cells with neurons. According to the disease maximum around day 14 p.i., we focused in these experiments on this time point after infection.

In \(\Delta\text{actA}\) LMova-infected NSE-OVA mice, CD45 leukocytes preferentially homed to the spinal cord, where they were mainly located in the anterior horn, with many CD3 T cells clustering intimately around the motor neurons (Fig. 6, A and C). In the brain, CD8 T cells were closely associated with OVA-expressing neurons (Fig. 6E). Both nonimmunized and immunized NSE-OVA mice showed an identical neuropathology that was more severe in immunized animals.
In contrast to NSE-OVA mice, a few CD45 leukocytes were diffusely scattered throughout the brain parenchyma of WT mice without any topographical preferences. Additionally, in the spinal cord of WT mice only very few CD45 leukocytes were present, mainly in the leptomeninges and in the vicinity of the central canal (Fig. 6B). In contrast to NSE-OVA mice, neurons of WT mice were neither associated with CD3 T cells in the spinal cord (Fig. 6D) nor with CD8 T cells in the brain parenchyma (Fig. 6F). Beyond day 14 p.i. (up to day 82 p.i.), neuropathology regressed in all experimental groups (data not shown).

Staining for LM demonstrated that at day 14 p.i. bacteria had been completely eliminated from the brain, indicating that OVA expression of neurons, but not OVA Ag derived from LM, induced the recruitment of CD8 T cells to neurons of NSE-OVA mice (data not shown).

Collectively, these experiments reveal NSE-OVA mice to develop a neurological disorder characterized by neuron-associated CD8 T cell infiltrates with neuropathology paralleling clinical disease activity.

**CD8 T cells induce autoimmunity in the CNS of NSE-OVA mice having been infected i.c. with ΔactA LMova**

To determine whether CD8 T cells play the anticipated important role in the development and course of disease, immunized NSE-OVA and WT mice were treated with rat IgG (1), anti-CD8 (2), anti-CD4 (3), or anti-CD4 + anti-CD8 Abs (4) before i.c. challenge infection with ΔactA LMova. To prevent a more severe listeriosis in T cell-depleted animals, all mice were perorally treated with an antibiotic (ampicillin), which kills LM, starting at day 3 after i.c. infection. NSE-OVA mice treated with rat IgG developed disease similar to NSE-OVA mice without Ab treatment (Fig. 7; for comparison, see Fig. 4). In contrast, anti-CD4 + anti-CD8...
treatment completely prevented the disease of NSE-OVA mice having been i.c. infected with ΔactA LMova. After CD8 T cell depletion, NSE-OVA mice also remained completely healthy, whereas anti-CD4 application had no effect on the onset, severity, duration, and incidence of the neurological disease (Fig. 7).

These experiments define CD8 T cells as decisive players in the induction of an autoimmune i.c. response and do not provide any evidence for a role of CD4 T cells in the induction and effector phase of the CD8 T cell response.

An adoptive transfer of OVA 257–264-specific CD8 T cells induces an aggravated course of the neurological disease in NSE-OVA mice infected i.c. with ΔactA LMova

The data presented above illustrate that i.c. infection with ΔactA LMova induces a CD8 T cell-mediated neurological disease, which can be boosted by immunization-mediated increase of the frequency of OVA 257–264 CD8 T cells. To further substantiate that CD8 T cells cause the autoimmune attack against OVA-expressing neurons and to evaluate whether an increase in the precursor frequency of OVA 257–264-specific CD8 T cells results in a more severe disease, we performed adoptive transfer experiments with purified OVA 257–264-specific CD8 OT-I T cells.

In the absence of an i.c. infection with ΔactA LMova, an adoptive transfer of OT-I T cells did not cause any clinical symptoms in both NSE-OVA and WT mice (data not shown). However, when naive OVA 257–264-specific CD8 T cells from OT-I mice were injected i.v. into NSE-OVA mice followed by an i.c. infection with ΔactA LMova 1 day later, these animals developed a severe neurological syndrome, which was significantly aggravated as compared with the disease in nonimmunized and immunized NSE-OVA mice after i.c. infection with ΔactA LM without OT-I transfer (days 7 and 11 p.i., p < 0.05; beyond day 21 p.i., p < 0.001; Fig. 8, A and B, compared with Figs. 3 and 4). The disease of NSE-OVA mice having received CD45.1+ OT-I T cells was characterized by an increased number of i.c. OVA 257–264-specific CD45.1+ CD8 T cells at days 3, 8, and 15 p.i. as compared with WT animals (Fig. 8C). In addition to donor-derived CD45.1+ OVA 257–264-specific CD8 T cells, the number of host-derived (endogenous) i.c. CD45.2+ OVA 257–264-specific CD8 T cells was increased in NSE-OVA mice (Fig. 8D), which further confirms our results in mice without T cell transfer (Fig. 5B). At days 8 and 15 p.i., absolute numbers of CD45.1+ OVA 257–264-specific CD8 T cells were at least 10-fold increased as compared with CD45.2+ OVA 257–264-specific CD8 T cells in both strains of mice (compare Fig. 8C and Fig. 8D), which is explained by the adoptive transfer of high numbers (2 × 10^7) of naive OT-I T cells. At day 8 p.i., ~93% of the total i.c. OVA 257–264-specific CD8 T cells are CD45.1+ and 7% are CD45.2+ in NSE-OVA mice. At day 15 p.i., 84% of the total i.c. OVA 257–264-specific CD8 T cells are CD45.1+ and 16% are CD45.2+ in NSE-OVA mice. In WT mice, ~95% of the total i.c. OVA 257–264-specific CD8 T cells are CD45.1+ and 5% are CD45.2+ in NSE-OVA mice at days 8 and 15 p.i. (Fig. 8, C and D). Clinical disease activity appeared to correlate with the frequency of i.c. CD45.1+ T cells (Fig. 8, A and C).

In NSE-OVA mice, OT-I T cell transfer and i.c. infection with ΔactA LMova induced symptoms reaching a maximum as early as day 7 p.i. and, importantly, mice exhibited a permanent residual neurological deficit with ataxia and disturbed coordination (score of 2) until the end of the observation period (Fig. 8, A and B).

In conclusion, these experiments demonstrate that the precursor frequency of OVA-specific CD8 T cells plays an important role for the course of the disease.

OVA 257–264-specific CD8 T cells are associated with MHC class I+ OVA-expressing neurons

Importantly, OT-I mice were on a CD45.1 background, which allowed tracking of the transferred T cells by CD45.1 staining. This enabled us to study whether donor-derived OVA 257–264-specific
CD8 T cells were associated with MHC class I\(^+\) OVA-expressing neurons in NSE-OVA mice after i.c. infection with \(\Delta actA\) LMova, and to study the fate of neurons in mice with maximal disease activity.

Adoptive transfer of OT-I cells resulted in an increased recruitment of CD45.1\(^+\) leukocytes to the brain of NSE-OVA mice after i.c. infection with \(\Delta actA\) LMova. At day 8 p.i., CD45.1\(^+\) cells, corresponding to the adoptively transferred CD8 T cells, were closely associated with OVA\(^+\) cells, which are exclusively neurons, as demonstrated in Fig. 1, and which also expressed MHC class I Ag (Fig. 9). Although the colocalization of CD3\(^+\) T cells with neurons was frequently observed in the spinal cord of NSE-OVA mice (Fig. 6, A and C), this was less frequent in the brain (Figs. 6F and 9). In contrast to NSE-OVA mice, only few CD45.1\(^+\) OT-I T cells were detectable in the CNS of WT mice having been infected with \(\Delta actA\) LMova, and these transferred cells were not found in association with neurons. At days 8 and 15 p.i., 20.9 and 28.9% of the neurons, respectively, in part associated with lymphocytes, in the anterior horn of the spinal cord of NSE-OVA mice were apoptotic, as evidenced by a basophilic nucleus, clumped nuclear chromatin, and shrinkage (Fig. 10, A, B, and D). Apoptosis of these neurons was further confirmed by TUNEL staining (Fig. 10D). In NSE-OVA mice, apoptosis of neurons in the brain was less prominent as compared with the spinal cord, paralleling the increased association of CD3 T cells with neurons in the spinal cord as opposed to the brain (data not shown). In WT mice, only low numbers of neurons in the anterior horn of the spinal cord became apoptotic (2.6 and 1.7% at days 8 and 15 p.i., respectively; Fig. 10, A, C, and E), resulting in a significantly increased number of apoptotic neurons in NSE-OVA mice as compared with WT mice (\(p < 0.0001\) and 0.00001 at days 8 and 15 p.i., respectively; Fig. 10A).

**Discussion**

Herein, we demonstrate in a model of “molecular identity”, in which transgenic mice express OVA specifically in neurons, that under appropriate conditions an infection of these animals with OVA-secreting LM causes an autoimmune attack against neurons. This new model enabled us to address the basic question of how pathogens might trigger an autoimmune attack against neurons. Mice with neuron-specific expression of OVA as autoantigen were healthy, fertile, and had a normal lifespan and architecture of the CNS without any pathology per se. Importantly, the widespread neuronal expression of the transgene did not render mice

![Image](http://www.jimmunol.org/DownloadedFrom)
tolerant to OVA, as demonstrated by the development of activated, Ag-specific CD8 T cells upon infection with ΔactA LMova. The undetectable OVA-specific CD4 T cell response after infection with attenuated LMova is most probably explained by the fact that OVA-specific CD4 T cell responses are very weak and subdominant compared with CD8 T cell responses against LLO190-201 and not by a tolerance of CD4 T cells against OVA. The absence of T cell tolerance against OVA corresponds to an exclusive transgene expression in the CNS, but not in the thymus and other organs. A defective expression of autoantigens in the thymus is also one important aspect of why autoreactive T cells are not eliminated in the thymus in humans and cause autoimmune diseases of the CNS (20). Conversely, tolerance against a self epitope can prevent the onset of a cerebral autoimmune disease induced by T cells cross-reacting between a pathogen-specific and a cerebral (self) autoantigen (21).

Cross-reactivity between a pathogen and neurons induced a CD8 T cell-mediated autoimmune disease manifesting as ataxic-paretic neurological syndrome with a 100% incidence. Thus, CD8 T cells can function as important effector cells in immune-mediated CNS diseases instead of playing only a minor regulatory role in brain inflammation or being only involved in pathogen clearance (4). In our model, CD8 T cells were in intimate contact with OVA-expressing MHC class I+ neurons in the spinal cord, the hippocampus, and the neocortex. The proinflammatory milieu caused by i.c. LM in concert with IFN-γ-producing T cells is likely responsible for the induction of MHC class I Ag on OVA-expressing neurons, thereby enabling their recognition by the autoantigen-specific CD8 T cells. The important role of IFN-γ for the induction of MHC class I expression of neurons has been demonstrated before (2), and neurons can be destroyed by CTLs (22). In fact, up to 30% of anterior horn spinal cord neurons were apoptotic and, remarkably, the destruction of neurons was most evident in anatomic regions perfectly fitting with the clinical symptoms. These findings are reminiscent of human Rasmussen’s encephalitis, in which CD8 T cells also kill neurons (6).

T cell depletion experiments illustrate that in contrast to CD8 T cells, CD4 T cells did not contribute to the disease, although NSE-OVA mice harbored increased numbers of LM-specific CD4 T cells in their brain after i.c. infection with ΔactA LMova. This is mainly explained by the fact that neurons expressed MHC class I but not MHC class II Ags (2) and also by the observation that the primary CD8 T cell response against LM is largely CD4 T cell independent (23).

The severity of clinical symptoms and the neuronal damage appeared to correlate with the frequency of OVA-specific CD8 T cells. Both a systemic immunization with ΔactA LMova as well as an adoptive transfer of high numbers of naïve OT-I CD8 T cells before i.c. challenge infection with ΔactA LMova aggravated the disease clinically and histopathologically. The observation that the frequency of autoreactive T cells determines disease severity is in accord with the concept that repetitive infections with pathogens sharing a cross-reactive T cell epitope with a cerebral autoantigen will increase the number of autoimmunity-inducing T cells and strongly increase the risk for the development of cerebral autoimmune disorders (24).

The induction of the autoimmune disease required the presence of the pathogen in the CNS, indicating that the local proinflammatory milieu plays an important role. Note that infection of the CNS by LM induced the recruitment of some activated macrophages to the CNS and an activation of microglial cells, which up-regulated MHC Ags (data not shown). Because local APCs are important for the induction of cerebral autoimmune diseases caused by molecular mimicry (25), these i.c. activated, potentially OVA-presenting APCs may also be important for the further activation of OVA-specific CD8 T cells. However, after i.c. infection LM rapidly spread to lymphatic organs, and priming and expansion of LM-specific T cells depend on DC in lymphatic organs. Thus, i.c. APCs might amplify but are unlikely to induce the OVA-specific CD8 T cell response in our model. These findings extend previous studies in experimental autoimmune encephalomyelitis and autoimmune keratitis, which also demonstrated that the proinflammatory milieu is important for the ensuing autoimmune disease (26-28). Additionally, in a model of virus-induced molecular mimicry, the induction of an immunopathologic self-reactive Th1 response against a cerebral Ag was dependent on the cerebral infection by the virus (21).

The importance of the proinflammatory i.c. milieu for the induction of the CD8 T cell-mediated disease in our model is further evidenced by the absence of clinical symptoms in mice after systemic infection with ΔactA LMova. In agreement with data published previously (11), an exclusively systemic infection with ΔactA LMova resulted in the recruitment of some OVA-specific CD8 T cells to the brain. However, this immunosurveillance was insufficient to up-regulate MHC class I Ags on neurons, to activate microglia, to recruit other leukocytes to the brain, and, despite the ubiquitous and continuous expression of OVA by neurons, to induce an autoimmune disease. These findings support the concept that molecular mimicry between a cerebral autoantigen and pathogens might trigger cerebral autoimmune disorders, but that the final pathogen-dependent stimulus might require a neurotropic pathogen (29). However, in mice expressing the nucleoprotein of the lymphocytic choriomeningitis virus in oligodendrocytes, a merely peripheral lymphocytic choriomeningitis virus infection was sufficient to induce an i.c. autoimmune reaction not requiring the presence of the pathogen in the CNS (30). This points either to specific features of the underlying infectious agent and/or to a hierarchy of protection among CNS target cells with neurons, which have a limited regenerative capacity, being more tightly protected than oligodendrocytes.

Importantly, the proinflammatory milieu induced by i.c. infection with OVA-negative LM was insufficient to induce a disease against OVA-expressing neurons. ELISPOT experiments confirmed that OVA-specific bystander CD8 T cells did not expand after i.c. infection with OVA-negative LM. These findings fit into the concept that an infection may cause the activation of bystander T cells, but that this activation, in general, is not sufficient to induce an autoimmune disease due to the extremely low number of T cells specific for a single autoantigen (31, 32). However, at least studies with transgenic T cells indicate that a strong increase in the frequency of bystander T cells specific for an autoantigen might result in an autoimmune attack after infection with an unrelated pathogen (33, 34).

The absence of clinical symptoms in mice infected i.c. with OVA-negative LM also illustrates that LM per se did not cause the clinical symptoms in NSE-OVA mice, and several additional lines argue against a direct destruction of neurons by LM. First, the attenuated LM mutant used in the present study only infects the ventricular system and adjacent structures, but not the parenchyma of the CNS (Ref. (14) and this study), whereas the disease provoked by OVA-specific CD8 T cells directly homed to OVA-expressing neurons in the parenchyma distant from the ventricular system. Second, in our T cell depletion experiments, mice infected i.c. with OVA-secreting LM were treated with ampicillin, which kills LM rapidly, to prevent development of a more severe cerebral listeriosis in the absence of T cells. This antibiotic abridgement of
cerebral listeriosis did not exert any effect on the CD8 T cell-mediated disease. Third, immunization of mice before i.c. challenge infection significantly reduced CFU and resulted in a more rapid elimination of LM from the brain, but aggravated the CD8 T cell-mediated neurological disease. Thus, although LM in principle has the capacity to infect neurons (12), it can be ruled out that LM caused or contributed to the neurological disease described in this study. The observations that neurological symptoms started and persisted after bacterial elimination from the brain in our model and that neuronal damage also became obvious beyond this time point may also be interesting for other autoimmune diseases of the CNS. Importantly, it has been shown before (35) that molecular mimicry between a neurotropic virus and a natural self Ag of oligodendrocytes (i.e., proteolipid protein) resulted in a rapid-onset, nonprogressive paralytic disease characterized by potent activation of self-reactive proteolipid protein-specific CD4 Th1 responses. Interestingly, in this model the persistence of the virus in the CNS had a strong influence on disease exacerbation induced by subsequent immunization with the mimicking peptide (35). Thus, in NSE-OVA mice a persisting infection with an OVA-expressing pathogen might induce a more severe or even progressive disease induced by a continuous destruction of neurons by cross-reactive CD8 T cells. This assumption is supported by the finding that the proinflammatory milieu in the CNS is an important parameter fostering the development of cerebral autoimmune disease induced by molecular mimicry between a pathogen and CNS structures (this study and Ref. (21)).

In our model, the neurological symptoms regressed over time. Because this was paralleled by a decline of i.c. OVA-specific CD8 T cells, the disappearance of the neuron-disturbing cell population most probably is the major factor contributing to the resolution of the disease. The exact mechanism of the reduction of i.c. OVA-specific CD8 T cells is unknown, but was most likely caused by apoptosis of these cells, which has been identified in several models of cerebral autoimmune diseases as the major pathway of T cell elimination. Additionally, the elimination of LM from the brain and the accompanying regressive proinflammatory milieu might contribute to the regressive disease activity at later stages of the disease. This indicates that specific features of the infectious disease affect the ensuing autoimmune disease.

Interestingly, nonimmunized and immunized NSE-OVA mice infected i.c. with ΔactA LMova clinically recovered completely. Although the clinical symptomatology is explained by the observed neuronal apoptosis, it is well known from clinical and neuropathological observations in disorders affecting the anterior horn of the spinal cord (e.g., in neurogenic muscular atrophy induced by poliomyelitis virus infection) that hyper trophy of undamaged muscles innervated by remaining intact spinal cord motor neurons may provide a protective role.

The observation that in our study transgenically expressed OVA induced a clinically relevant neurological disease after i.c. infection with OVA-transgenic LM may also be of relevance for vector-based gene therapies of CNS disorders. There is good evidence that in vector-based gene therapies of neurological disorders T cells might attack CNS cells, which express the transgene (36). Such an immune response might be massively amplified by infectious pathogens, which invade the CNS and share a cross-reactive epitope with the transgene applied therapeutically.

Acknowledgments
We gratefully acknowledge the expert technical assistance of Marianna Carirov. We thank Dr. Iain L. Campbell and Dr. F. Carbene for kindly providing the NSElacZ and pBlueRIP-TIR/OVA plasmid, respectively, and Dr. Hans Lassmann for helpful discussion.

Disclosures
The authors have no financial conflicts of interest.

References


