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Cutting Edge

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Cutting Edge: Multiple Sclerosis-Like Lesions Induced by Effector CD8 T Cells Recognizing a Sequestered Antigen on Oligodendrocytes[1]

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CD8 T cells are emerging as important players in multiple sclerosis (MS) pathogenesis, although their direct contribution to tissue damage is still debated. To assess whether autoreactive CD8 T cells can contribute to the pronounced loss of oligodendrocytes observed in MS plaques, we generated mice in which the model Ag *influenza* hemagglutinin is selectively expressed in oligodendrocytes. Transfer of preactivated hemagglutinin-specific CD8 T cells led to inflammatory lesions in the optic nerve, spinal cord, and brain. These lesions, associating CD8 T cell infiltration with focal loss of oligodendrocytes, demyelination, and microglia activation, were very reminiscent of active MS lesions. Thus, our study demonstrates the potential of CD8 T cells to induce oligodendrocyte lysis in vivo as a likely consequence of direct Ag-recognition. These results provide new insights with regard to CNS tissue damage mediated by CD8 T cells and for understanding the role of CD8 T cells in MS. *The Journal of Immunology,* **2008, 181: 1617–1621.**

The immune effector mechanisms contributing to tissue damage in multiple sclerosis (MS)[4] are only partially elucidated. The heterogeneity of MS likely reflects a varying contribution of humoral factors, T cell subsets, and activated macrophages/microglia. Indirect evidence suggests that the role of CD8 T cells has been largely underestimated to date. Indeed, CD8 T cells accumulate within active MS lesions where they often outnumber CD4 T cells (1–3). These CNS-infiltrating CD8, but not CD4, T cells exhibit oligoclonal expansion, a likely consequence of their local Ag-driven activation (3). Moreover, myelin-reactive cytotoxic CD8 T cells have been identified in MS patients, sometimes more frequently than in controls (4, 5). Finally, active MS plaques exhibit MHC class-I expression on CNS oligodendrocytes and neurons/axons (6), which become potential targets of CD8 T cells. Altogether, these data suggest that CD8 T cells may be mediators rather than regulators of CNS inflammation and damage in MS.

Although the loss of oligodendrocytes, the CNS myelin-producing cells, is a key feature of MS lesions, the precise contribution of CD8 T cells to oligodendrocyte death and to CNS demyelination is unknown. It has been shown in vitro that myelin-specific CD8 T cells can kill isolated HLA-matched oligodendrocytes (4), but data in vivo are less compelling. Previous studies have shown that CD8 T cells are necessary for the development of a full-blown pathology in animal models of neuroinflammation, but their Ag specificity is still unknown (2, 7, 8). Similarly, a role for CD8 T cells in demyelination has been clearly illustrated in viral models of CNS inflammation, but the mechanisms involved have remained contentious (9, 10). Myelin-specific CD8 T cells can adoptively transfer autoimmune encephalomyelitis but the lesions were reminiscent of ischemic injury with the demyelination associated with more global tissue damage, and few CD8 T cells actually infiltrated the CNS parenchyma (11, 12). Therefore, the direct effect of CD8 T cells on oligodendrocytes and myelin in vivo is not clear.

In this study, to test whether CNS-infiltrating CD8 T cells can directly induce oligodendrocyte death and demyelination, we developed a mouse model combining selective expression of *influenza* hemagglutinin (HA) as a neo-self-Ag in oligodendrocytes with transgenic mice expressing a HA-specific TCR on CD8 T cells.

Materials and Methods

Mice, generation, and characterization of the Rosa-Stop-HA knock-in mice

The CL4-TCR mouse expresses a TCR specific for the *influenza* virus $HA_{512–520}$ peptide on most CD8 T cells (13). The MOGi-Cre knock-in mouse

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[4] Abbreviations used in this paper: MS, multiple sclerosis; CAII, carbonic anhydrase II; CNPase, cyclic nucleotide 3′-phosphodiesterase; DKI, double knock-in; GrB, granzyme B; HA, hemagglutinin; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis.

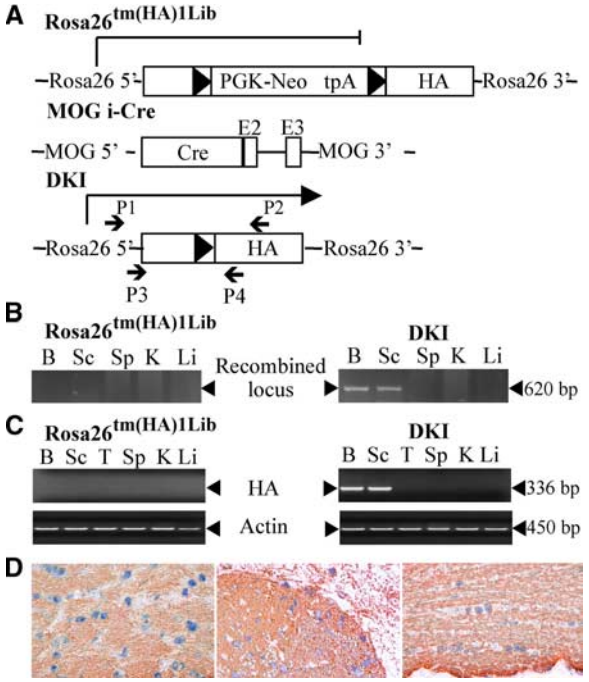

FIGURE 1. Generation and characterization of DKI mice. *A*, Schematic representation of the knock-in mice. *Top row*, The targeted Rosa26 locus in the Rosa26$^{tm(HA)1Libl}$ mice contains the LoxP-flanked Stop cassette and the HA sequence. Filled triangles indicate the LoxP sites. *Middle row*, The Cre sequence inserted in the *mog* gene in MOGi-Cre mice. *Bottom row*, The Cre-mediated recombination of the Rosa26 locus in DKI mice allows transcription of HA. P1, P2, P3, and P4 represent the position of the primers used. *B*, Cre-mediated recombination was assessed by PCR on genomic DNA from different organs using P1 and P2 primers. One representative experiment from a total of three is shown. *C*, Transcription of HA in different tissues was assessed by nested RT-PCR using P1/P2 then P3/P4 primers. Actin was used to evaluate cDNA quality. No HA signal was obtained when the reverse-transcription step was omitted. Similar results were obtained in three different mice per group. Tissue abbreviations: B, brain; Sc, spinal cord; T, thymus; Sp, spleen; K, kidney; Li, liver. *D*, Double immunostaining detection of *lacZ* expression (blue) in oligodendrocytes (CNPase; red) of a R26R reporter mouse crossed with a MOGi-Cre mouse. White matter tracts in the corpus callosum (*left*), spinal cord (*middle*), and optic nerve (*right*) (original magnification: ×260) are shown.

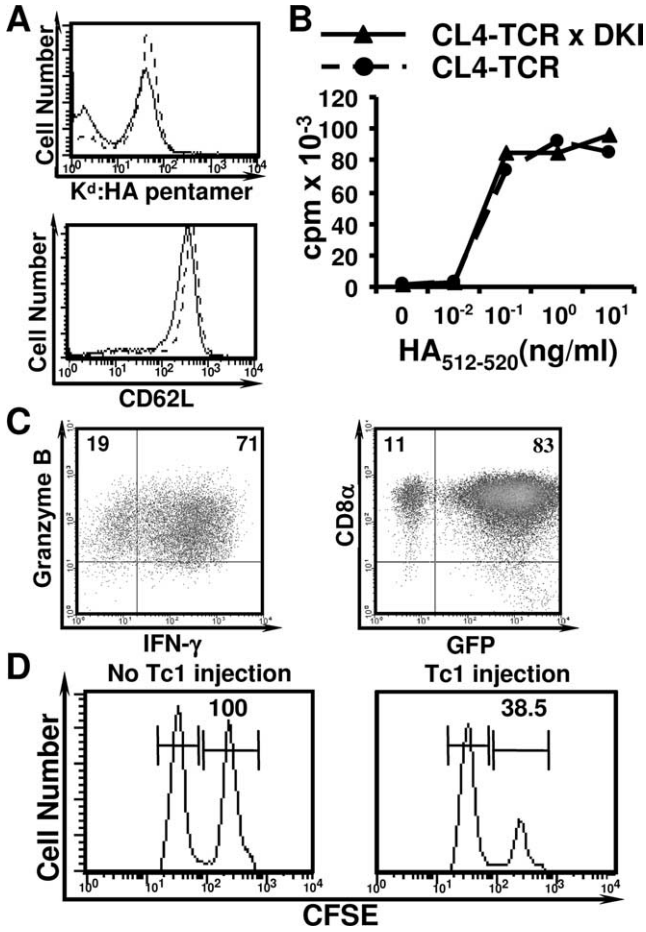

FIGURE 2. CD8 T cell indifference in DKI mice and characterization of effector CD8 T cells. *A*, Gated CD8$^+$ CD4$^-$ lymph node cells from CL4-TCR (dotted line) or DKI × CL4-TCR (solid line) mice were stained with K^d:HA$_{512–520}$ pentamer (*top*) or anti-CD62L mAb (*bottom*). Similar data were obtained for seven mice per group. *B*, Proliferation of purified CD8$^+$ T cells from CL4-TCR (dotted line) or DKI × CL4-TCR mice (black line) stimulated with HA$_{512–520}$ peptide. These results are representative of five independent experiments. *C*, Following a 6-day culture, the HA-specific CD8 T cells from CL4-TCR mice differentiated into IFN-γ-producing, granzyme B$^+$ T cells (*left*). Following transduction with a retroviral vector encoding GFP, most of the HA-specific Tc1 cells were brightly fluorescent at day 6, just before their adoptive transfer (*right*). *D*, The HA-specific Tc1 cells exhibit Ag-specific cytotoxicity in vivo. The histograms are gated on CFSE-labeled cells; the ratios of CFSE^{high} to CFSE^{low} peaks indicated that the Ag-specific lysis in this experiment was 61.5 ± 2.8% (n = 3 mice/group).

(where MOG is myelin oligodendrocyte glycoprotein) (14) was backcrossed >10 times on the BALB/c background.

A conditional expression cassette, encompassing the open reading frame of transmembrane HA placed 3′ of a Stop sequence (*neoR* and a trimer of SV40 polyadenylation sites) flanked by LoxP sites (Fig. 1*A*), was inserted into the *Pac*I-*Asc*I sites of the pROSA26PA vector (15). This gene-targeting vector was then electroporated into 129SV embryonic stem cells. Two embryonic stem cell clones exhibiting homologous recombination were injected into C57BL/6 blastocysts to generate chimeras that transmitted the Rosa-Stop-HA allele. These knock-in mice, referred to as Rosa26$^{tm(HA)1Libl}$, were crossed seven times onto the BALB/c background.

Cre-mediated recombination of the Rosa26-Stop-HA locus was assessed by PCR on genomic DNA from different organs. To analyze HA expression, RNA was extracted using TRIzol, contaminating DNA was removed by DNaseI digestion (Promega), and a quantitative RT-PCR or a nested RT-PCR approach was followed.

In vitro differentiation, GFP transduction, adoptive transfer of HA-specific Tc1 cells, and in vivo cytotoxicity

HA-specific Tc1 cells were generated as described and routinely contained >98% pure CD8$^+$CD3$^+$Vβ8.2$^+$ T cells (13). GFP transduction of the HA-specific Tc1 cells was performed using the pLGFPSN retroviral vector and the GP+E-86 packaging cell line. At day 6, living CD8 T cells were collected by Ficoll density separation and naive CD8 T cells or Tc1 cells were injected i.v. into immunocompetent recipient mice. Mice were assessed daily. For in vivo cytotoxicity, BALB/c mice were injected or not injected with 2 × 10^6 HA-specific Tc1 cells. The next day they all received 20 × 10^6 HA-pulsed and 20 × 10^6 control peptide-pulsed splenocytes stained with a high (CFSE^{high}) or low (CFSE^{low}) concentration of CFSE, respectively. After 24 h, spleens from the recipient mice were analyzed by flow cytometry.

Histopathology

At the specified time points, mice were perfused with 4% paraformaldehyde in PBS. Tissues were removed and embedded in paraffin. Five micrometer-thick sections were stained with H&E and Luxol fast blue/periodic acid Schiff myelin stain. Immunohistological staining and confocal laser microscope analyses were performed as described previously (13, 14).

Results and Discussion

Naive HA-specific CD8 T cells are "indifferent" in mice that express HA as an oligodendroglial neo-self Ag

For this study, we designed a mouse model system ensuring specific expression of HA in oligodendrocytes by using a double

Table I. *Summary of the clinical and histological signs of Tc1-injected mice*

Day of Sacrifice	Genotype (No. of Mice)	Weight Loss at Day 9[a]	Spinal Cord		Optic Nerve	
			Inflammation	Demyelination	Inflammation	Demyelination
Day 2.5	DKI ($n = 3$)	NA[b]	0/3	0/3	0/3	0/3
	Control mice[c] ($n = 3$)	NA	0/3	0/3	0/3	0/3
Day 5	DKI ($n = 4$)	NA	4/4	0/4	3/3	1/3
	Control mice ($n = 3$)	NA	0/3	0/3	0/3	0/3
Day 9	DKI ($n = 13$)	5/13	9/11	0/11	9/9	5/9
	Control mice ($n = 18$)	0/18	0/16	0/16	0/16	0/16
Day 18	DKI ($n = 5$)	3/5	5/5	1/5	4/4	4/4
	Control mice ($n = 5$)	0/5	0/2	0/2	0/2	0/2
Day 28	DKI ($n = 5$)	2/5	5/5	3/5	4/4	4/4
	Control mice ($n = 9$)	1/9	0/9	0/9	0/9	0/9
Day 56	DKI ($n = 3$)	1/3	0/3[d]	0/3	0/3[d]	0/3[e]
	Control mice ($n = 5$)	0/5	0/5	0/5	0/5	0/5

[a] Body weight <95% of the weight before Tc1 injection.
[b] NA, Not applicable.
[c] Rosa26$^{tm(HA)1Libl}$, MOGi-Cre, and nontransgenic littermates.
[d] Absence of perivascular inflammatory infiltrates but some diffuse tissue infiltration by $CD3^+$ cells.
[e] Focal areas of reduced myelin density with thin myelinated sheaths, suggesting remyelination.

knock-in (DKI) approach. First, we generated mice, referred to as Rosa26$^{tm(HA)1Libl}$, in which the HA coding sequence was introduced in the ubiquitously active *Rosa26* locus but where HA transcription was prevented by an upstream LoxP-flanked Stop cassette (Fig. 1*A*). The Rosa26$^{tm(HA)1Libl}$ mice were then crossed with the MOGi-Cre mice (14), which express Cre specifically in oligodendrocytes (Fig. 1*A*). The resulting DKI mice excise the Stop cassette due to MOG-controlled Cre expression, leading to restricted HA expression to oligodendrocytes. The DKI mice exhibited no spontaneous phenotype.

PCR analyses of the genomic DNA of DKI and Rosa26$^{tm(HA)1Libl}$ mice confirmed that Cre-mediated recombination occurred only in the CNS of DKI mice (Fig. 1*B*). As a result, HA transcripts were detected by quantitative RT-PCR only in the brain, spinal cord, and optic nerve of DKI mice (data not shown), although HA protein expression in the CNS was below detection levels using immunohistochemistry. Moreover, using a sensitive nested RT-PCR approach, HA RNA was undetectable in extra-neurological tissues (Fig. 1*C*). Further indirect evidence of oligodendrocyte specificity of the transgene expression was provided upon crossing MOGi-Cre mice with the R26R reporter mice, which are similar in design to the Rosa26$^{tm(HA)1Libl}$ mice apart from the substitution of *HA* by *lacZ*. In these mice, LacZ expression was only detected in oligodendrocytes (Fig. 1*D*).

Interestingly, we did not observe any neurological signs upon crossing the DKI mice with CL4-TCR mice (25 CL4-TCR × DKI mice followed for up to 9 mo). Moreover, we did not detect any TCR down-regulation or deletion of HA-specific CD8 T cells, as assessed by K^d:$HA_{512–520}$ pentamer staining (Fig. 2*A*). Rather, peripheral CD8 T cells appeared "indifferent" to HA, as they exhibited mostly a naive phenotype ($CD25^-CD69^-CD62L^{high}CD44^{intermediate}$ and retained full proliferative capacity in response to the $HA_{512–520}$ peptide (Fig. 2, *A* and *B*). These data are consistent with a lack of expression and probably also of cross-presentation of HA in the lymphoid organs of DKI mice. Therefore, the MOG-driven HA protein behaves as a sequestered oligodendrocyte Ag in DKI mice.

Transfer of effector HA-specific CD8 T cells into HA-expressing mice results in CNS inflammation and demyelination

We then decided to test whether effector CD8 T cells can mediate oligodendrocyte cell death and demyelination in vivo. Effector T cells were first generated by in vitro activation of K^d:$HA_{512–520}$ pentamer$^+$ CD8 T cells obtained from CL4-TCR mice using HA peptide, IL-2, and IL-12. The resulting Tc1 cells produce large amounts of granzyme B (GrB) and IFN-γ (Fig. 2*C*, *left*). These cells exhibit potent cytotoxicity to HA-loaded target cells in vivo (Fig. 2*D*).

Next, we transferred these HA-specific Tc1 cells into DKI and control mice. Following i.v. injection of 3×10^7 HA-specific Tc1 cells, but not naive HA-specific CD8 T cells, >40% of the DKI mice developed an overt monophasic disease peaking at day 8–10 and waning by 4 wk posttransfer. The clinical manifestations included weight loss and, in the more severe cases, tremors, reduced mobility, and difficulty to right when overturned without overt paralysis (Table I). In the littermate controls, only one animal exhibited weight loss and none developed any neurological signs ($p = 0.0004$). This Ag-specific, CD8-mediated disease also provides strong evidence for functional HA protein expression in vivo in DKI mice.

Upon histological analysis, all DKI mice injected with Tc1 cells demonstrated clear CNS pathology from day 5 onwards (Table I). Parenchymal infiltrates were never observed in control littermates injected in parallel with HA-specific Tc1 cells. The pathology of DKI mice was dominant in the optic nerve (Figs. 3 and 4) and spinal cord (Fig. 4) but also affected frequently the cerebellum, fornix, and periventricular areas of the brain. Inflammatory lesions were never found in the peripheral nervous system or in non-nervous tissues. Quantification revealed that T cell infiltration started on day 5 in the spinal cord, reached its peak on day 9, and declined by day 28 (Fig. 5*A*). On

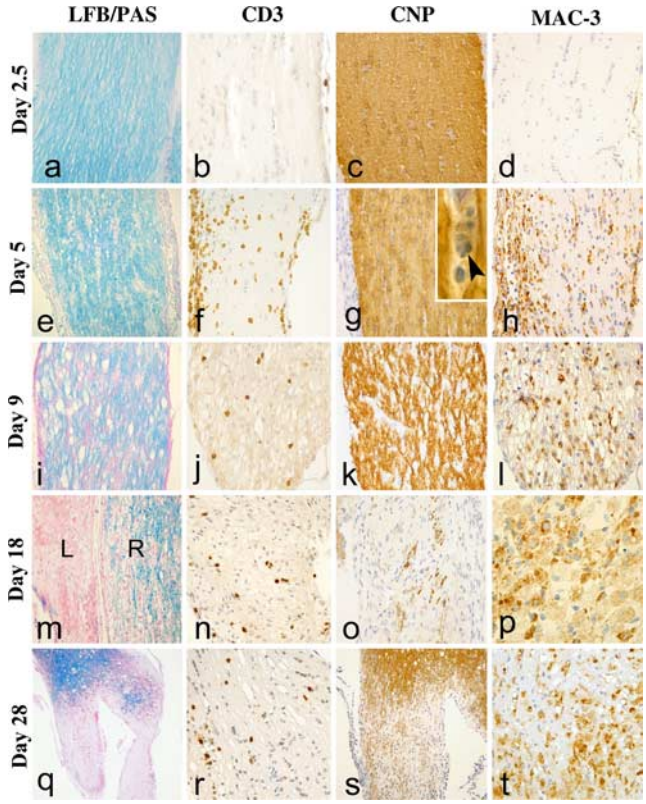

FIGURE 3. Optic nerve pathology in DKI mice following injection of HA-specific Tc1 cells. *a–d* (day 2.5), Staining shows the absence of myelin pathology (Luxol fast blue/periodic acid Schiff and CNPase) and of T cell infiltration (CD3). *e–h* (day 5), Large numbers of $CD3^+$ cells (*f*) and activated microglia (*h*) have infiltrated the parenchyma. The myelin stain shows some vacuolization (*e*) but no demyelination (*e* and *g*). Oligodendrocytes with condensed nuclei (*inset* in *g*) are found, indicating apoptosis. *i–l* (day 9), Myelin stains (*i* and *k*) reveal vacuolization and some demyelination. The number of T cells (*j*) has declined (see Fig. 5*A*, *right*), but the MAC-3 staining (*l*) shows phagocytic microglia. *m–p* (day 18), Extensive demyelination is seen in the left (L) optic nerve (*m*). Demyelination is ongoing in the right (R) optic nerve, with myelin degradation products in PAS^+ macrophages. T cells (*n*) and large phagocytic cells (*p*) are still present. The CNPase staining (*o*) shows some remaining oligodendrocytes in the left optic nerve. *q–t* (day 28), Both optic nerves show extensive demyelination (*q*) and ongoing inflammation (*r* and t). CNPase staining shows some reactivity in the left optic nerve (*s*), which may indicate remyelination. All figures have a ×100 original magnification with the exception of *q* (×26), *s* (×70), and the *inset* in *g* (×1000).

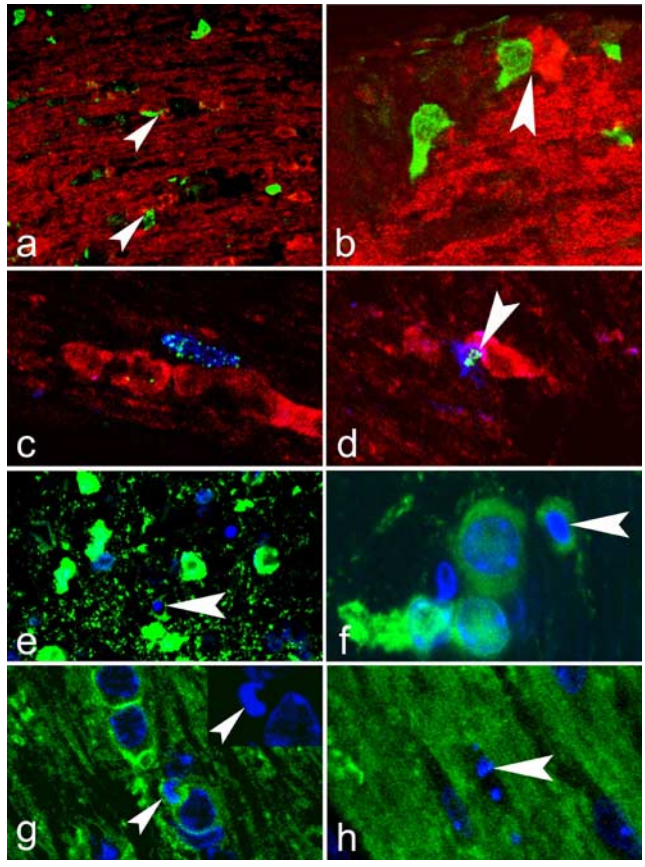

FIGURE 4. Detection of CD8-mediated oligodendrocyte killing in the spinal cords and optic nerves of DKI animals on day 9. *a* and *b*, GFP (green) and CNPase (red) double staining (×450). *a*, Some GFP^+ HA-specific CD8 T cells lie in close apposition to oligodendrocytes (arrowheads) in the optic nerve. *b*, An HA-specific CD8 T cell in contact (arrowhead) with an oligodendrocyte. *c* and *d*, Triple staining for GrB (green), GFP (blue), and CAII (red) (×1400). *c*, A GrB^+ HA-specific CD8 T cell is next to a row of oligodendrocytes. *d*, Another example of a HA-specific T cell in close apposition to an oligodendrocyte, with its GrB-containing granules polarized toward the surface of the oligodendrocyte. *e–h*, Staining for oligodendrocytes (green), with TO-PRO-3 nuclear counterstain. *e*, One of the $CAII^+$ oligodendrocytes shows nuclear condensation (arrowhead), indicative of apoptosis (×850). *f*, Apoptosis (arrowhead) of a $CAII^+$ oligodendrocyte in the optic nerve (×2000). *g*, Nuclear condensation (arrowhead) in a $CNPase^+$ oligodendrocyte. The *inset* shows this condensed nucleus without CNPase staining (×1500). *h*, An oligodendrocyte with nuclear condensation and fragmentation into apoptotic bodies (×1800).

day 56, there was only mild diffuse infiltration of the CNS by $CD3^+$ T cells. In the optic nerves we found focal areas with reduced myelin density and thin myelin sheaths, suggesting remyelination.

We also transferred GFP-transduced Tc1 cells (Fig. 2*C*, *right*), allowing us to trace the CNS-invading, HA-specific CD8 T cells. Staining for GFP and GrB revealed that HA-specific Tc1 cells containing GrB^+ granules were detected within CNS lesions of DKI mice (Fig. 4*c*). Some of the GrB^+ T cells were found in close apposition to carbonic anhydrase II (CAII) or cyclic nucleotide 3′-phosphodiesterase$^+$ (CNPase) oligodendrocytes (Fig. 4, *c* and *d*). We also detected Tc1 cells with polarized GrB^+ granules facing an oligodendrocyte, suggesting directed degranulation (Fig. 4*d*). In the inflamed lesions, MHC class I (β_2-microglobulin) expression was detected on oligodendrocytes (Fig. 5*B*). The CD8 T cell infiltration was associated with focal activation of microglia (Fig. 3, *h*, *l*, *p*, and *t*). Besides the presence of CD8 T cells and activated microglial cells, lesions consisted of CD4 T cells (1–13% of T cells depending on the time point) and small numbers of $B220^+$ B cells (mostly in the perivascular space).

Oligodendrocyte and myelin pathology was studied on sections stained for Luxol fast blue, CNPase, and CAII (Figs. 3 and 4). Oligodendrocyte apoptosis, detected by nuclear condensation (Fig. 3*g* and Fig. 4, *e–h*), was present at early time points in optic nerves and spinal cords of DKI recipients. In the optic nerve of DKI mice, the number of apoptotic oligodendrocytes was 6.9 ± 3.5 per section on day 5 and 15.3 ± 4.8 on day 9, whereas they were virtually absent in control mice. On day 5, some vacuolization and early demyelination was found in the optic nerve (Fig. 3*e*). From day 9 on, most of the animals exhibited extensive demyelination in the optic nerve (Fig. 3, *i*, *m*, and *q*) and more limited demyelination in the brain and spinal

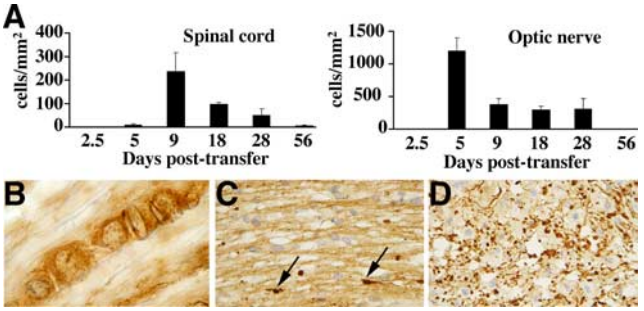

FIGURE 5. T cell infiltration and axonal damage in the CNS of DKI mice. *A*, Quantification of $CD3^+$ T cells in the spinal cord and optic nerve of DKI mice at different time points after Tc1 cell injection (Table I). *B*, β_2-Microglobulin expression by oligodendrocytes in the spinal cord on day 9 (×650). *C* and *D*, Axonal injury in demyelinating lesions of the optic nerve, detected with SMI311 staining nonphosphorylated neurofilaments (×250). By day 9 (*C*) only some axonal spheroids are present (arrows), whereas at day 18 (*D*) massive axonal destruction is observed.

cord. In addition, staining for nonphosphorylated neurofilament revealed severe axonal damage in active demyelinating lesions by day 19 (Fig. 5, *C* and *D*). Importantly, Ab-mediated depletion of CD4 T cells in vivo had no impact on the severity of CNS inflammation and demyelination (data not shown), excluding their pathogenic contribution in this model. Collectively, our data show that demyelinating lesions, sharing many of the attributes of an active MS plaque, can arise when CD8 T cells target oligodendrocytes.

The experimental system described here allows the clear assessment of the individual role of cytotoxic CD8 T cells by uncoupling them from other adaptive immune mechanisms. Genetic GFP labeling of the transferred CD8 T cells permitted the unequivocal tracing of the HA-specific CTLs in situ. Strikingly, we show that numerous HA-specific CD8 T cells enter the CNS and optic nerve parenchyma and colocalize with oligodendrocytes, with occasional figures of tight apposition between the two cell types. These data strongly suggest that direct cell contact-mediated cytotoxicity plays a central role in oligodendrocyte death and demyelination. It is, however, possible that soluble inflammatory mediators synergize to induce tissue lesions.

In conclusion, we generated a mouse model to study the contribution of T cell subsets on CNS tissue damage. An analogous transgenic model has been recently used to investigate CD4 T cell reactivity to OVA expressed specifically in oligodendrocytes (16). We focused on oligodendrocyte-specific CD8 T cells because little in vivo information is currently available regarding the pathogenicity of this subset and because both CD8 T cell infiltration and loss of oligodendrocytes are essential features of MS lesions. The novel finding provided by this study is that effector CD8 T cells exhibit a potent deleterious effect on oligodendrocytes, resulting in an inflammatory demyelinating pathology resembling active MS lesions. The exclusive expression of HA in oligodendrocytes is very reminiscent of some myelin self-Ags such as MOG, which are not (or barely) expressed in the lymphoid tissue and therefore fail to tolerize the T cell repertoire (17). This carries an obvious risk of activation of autoreactive CD8 T cells with subsequent development of autoimmunity. Collectively, these data reinforce the idea that CD8 T cells represent relevant therapeutic targets in MS (1, 2).

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Disclosures

The authors have no financial conflict of interest.

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