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CUTTING EDGE

Cutting Edge: Cross-Presented Intracranial Antigen Primes CD8$^+$ T Cells[1]

Lisa Walter and Matthew L. Albert[2]*†*

The CNS is considered immune privileged due to the blood-brain barrier and the absence of conventional lymphatics. Nonetheless, T cell immune responses specific for CNS Ag have been documented. Where these events are initiated and what cellular mechanisms are involved remain unknown. In this study, we established an experimental mouse model to evaluate the requirements for priming CD8$^+$ T cells following the cross-presentation of intracranial Ag. Surprisingly, we find that even with a damaged blood-brain barrier, Ag presentation occurs in regional lymph nodes and not within the CNS itself. Only once the responding cells have expanded can they traffic to the site of CNS injury. Cross-presentation of intracranial Ag is efficient and the subsequent priming of CD8$^+$ T cells is dependent on CD4$^+$ T cell help and CD40 signaling in host APCs. Our findings have important implications for the initiation of T cell immune responses toward CNS Ags. The Journal of Immunology,* 2007, 178: 6038–6042.

The presentation of intracranial (IC)[3] Ags by the immune system to generate a T cell-priming response remains unclear. The CNS, which consists of the brain and spinal cord, has been considered to be immune-privileged, a concept that is supported by the lack of conventional lymphatics and the presence of the blood-brain barrier (1, 2). Nonetheless, considerable data exist to support a role for CD4$^+$ T cells in the initiation and progression of experimental autoimmune encephalomyelitis as well as other CNS pathologies (3). In particular, it has been shown that dendritic cells (DCs) are critical for the priming of CD4$^+$ T cells specific for brain Ag (4), but where the initial priming event occurs remains controversial. Although human and monkey data indicate that Ag presentation may be outside the CNS as supported by the observation that APCs harbor CNS proteins in cervical lymph nodes (LNs) (5), recent mouse data supports initial CD4$^+$ T cell engagement occurring within the CNS (6).

With respect to priming CD8$^+$ T cells, less is understood, but some models have demonstrated that it is possible to initiate a CD8$^+$ T cell response specific to intracranial Ag (7, 8) as well as to CNS infection (9). In these studies, the CD8$^+$ T cell immunity was initiated by adoptive transfer of the T cells themselves (10) or through injection of DCs loaded with peptide (8). Finally, there exists evidence that APCs capture and present Ag derived from tumor cells injected into brain cerebrum, resulting in the imprinting of T cells with a CNS-homing phenotype (11). Together, these data suggest that T cell priming to IC Ags is possible.

In this study, we used an experimental model in which cell-associated IC Ag must be cross-presented by host APCs for the initiation of immunity. We address the site of T cell encounter with APCs that have captured IC Ag, and, strikingly, although the blood-brain barrier was disrupted due to injection, expansion of responding T cells occurred in cervical LNs and not in brain parenchyma. T cell engagement resulted in efficient cross-priming of the endogenous CD8$^+$ T cell repertoire and using transgenic OVA-specific CD8$^+$ T cells (OT-1) to monitor brain infiltration, we show that priming in the LNs is a prerequisite for T cell entry into the CNS, even in the presence of additional Ag-loaded APCs. Finally, we show that CD4$^+$ T cell help is required for the CD40-dependent licensing of APCs cross-presenting intracranial Ag. We conclude that initial CD8$^+$ T cell-priming events occur outside the CNS and that Ag presentation in situ in the brain happens only after T cells have been primed in the periphery.

Materials and Methods

Mice, cell lines, Abs, and reagents

C57BL/6J (WT) mice were obtained from Charles River. Ptprc^{a}Pepc^b/BoyJ (CD45.1), Tg(TcraTcrb)1100Mjb (OT-1), *Cd40^{tm1Kik}* (CD40$^{-/-}$), *Il6^{tm1Kopf}* (IL-6$^{-/-}$) and *Il12a^{tm1Jm}* (IL-12p35$^{-/-}$) mice were obtained from The Jackson Laboratory, bred in-house, and maintained in a specific pathogen-free facility. Mice expressing membrane-bound full-length OVA under an actin promoter were a gift from Dr. M. Jenkins (University of Minnesota, Minneapolis, MN) (12) and the cross onto the H-2K$^{b-/-}$ was performed by Dr. S. Schoenberger (La Jolla Allergy and Immunology, La Jolla, CA) (13). FACS Abs were

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[3] Abbreviations used in this paper: IC, intracranial(ly); DC, dendritic cell; LN, lymph node; WT, wild type; IF, intrafootpad.

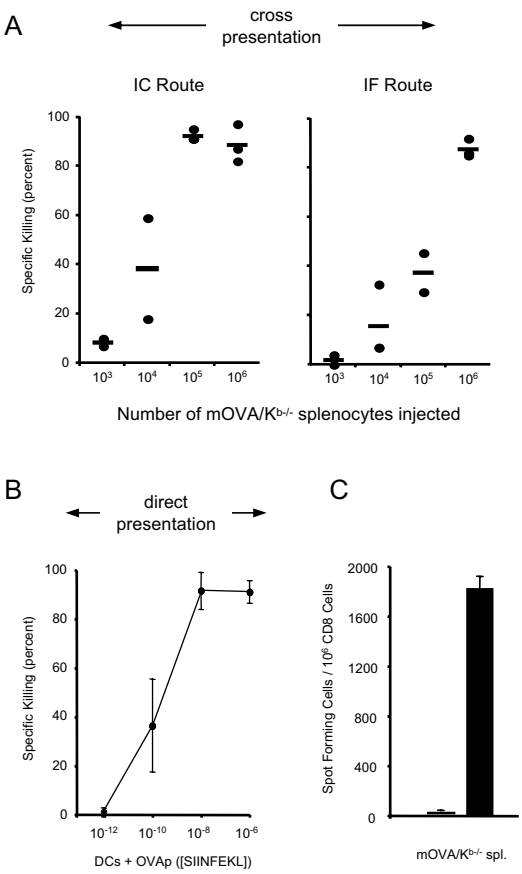

FIGURE 1. Cross-presentation of IC Ag induces CD8$^+$ T cell priming. *A,* WT mice were given increasing numbers of mOVA/K$^{b-/-}$ splenocytes via the IC or IF routes, and an in vivo killing assay was performed. Individual mice are represented, and bars indicate mean values. Data are representative of two experiments. *B,* DCs were pulsed with OVAp (SIINFEKL) and administered IC into WT mice. In vivo killing activity was evaluated on day 6. Data points are pooled from multiple experiments and are mean values ± SD, $n = 3$–13 mice/condition. *C,* Following IC administration of 10^6 mOVA/K$^{b-/-}$ splenocytes, an ex vivo IFN-γ ELISPOT assay was performed using purified CD8$^+$ T cells restimulated by peptide-pulsed (■) or unpulsed targets (□). Data are mean values of triplicate wells + SD and are representative of six mice tested in three experiments.

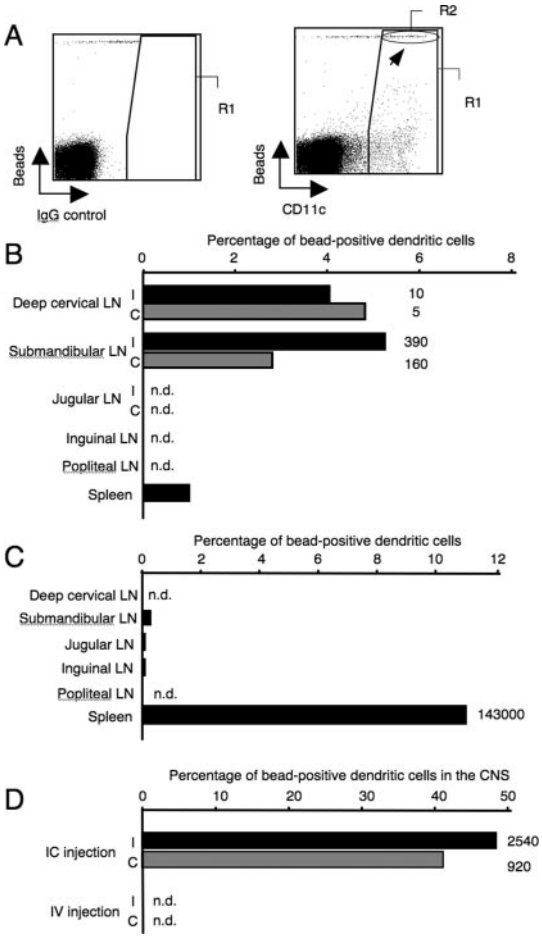

FIGURE 2. IC-administered beads traffic to specific regional LNs. *A,* The gating strategy is shown for determining the percentage of DCs that have captured fluorescent beads. CD11c$^+$ DCs were identified (gate R1) and further analyzed for the presence of beads (shown in gate R2). Beads were administered IC (*B*) or i.v. (*C*) and 2 days later organs were examined for bead-positive cells using the gating strategy shown. Bead-positive DCs were determined by dividing the number of events in R2 by total DC numbers (events in R1). Numeric values indicate the absolute number of CD11c$^+$ bead-positive events per organ. For relevant sites, we report data from ipsilateral (I) and contralateral (C) LNs. *D,* Beads were administered IC or i.v. and the percentage of bead-positive DCs present in the left and right cerebral brain hemispheres are shown. Data are taken from individual mice and is representative of four mice per condition. n.d., Not detected.

obtained from BD Biosciences. GK1.5 Ab was grown from a hybridoma (American Type Culture Collection).

Ag administration

IC Ag was administered by injection into the right cerebrum. A 20-ml Hamilton syringe attached to a 25-gauge needle and penetrating depth controller was used for IC, s.c., and intrafootpad (IF) injections. Intravenous injections were performed retro-orbitally. Two-micrometer fluorescent carboxylate polystyrene microspheres were used in bead studies (Polysciences). Unless otherwise stated, 10^6 splenocytes or brain cells were used.

Monitoring CD8$^+$ T cell activation

For in vivo cytotoxicity assays, target cells were prepared from WT splenocytes and split into two populations. One part was labeled with 5 μM CFSE (CFSE^{high}) and pulsed with 10 μM OVAp and the other was labeled with 0.5 μM CFSE (CFSE^{low}). Target cells were washed, mixed at a 1:1 ratio, and 10^7 cells were injected i.v. After 20 h, splenocytes of recipient mice were isolated and killing was assessed: percent specific killing = $100 \times (1 - [\text{CFSE}^{high}/\text{CFSE}^{low}]_{sample}/[\text{CFSE}^{high}/\text{CFSE}^{low}]_{control})$. To determine IFN-γ production in primed CD8$^+$ T cells, ELISPOT assays were performed. CD8$^+$ T cells were purified using MACS technology (Miltenyi Biotec) as described previously (14). Enumeration of spot-forming cells was performed by an independent service (Zellnet Consulting). In vivo proliferation of OT-1 CD8$^+$ T cells was evaluated using CFSE-labeled cells isolated from CD45.1 OT-1 mice.

Cell preparation and flow cytometry

Animals were perfused with PBS, and lymphocytes were prepared from brains, LNs, and spleens. Cerebellum, spinal cord, and olfactory bulbs were removed from each brain, and cerebrums were digested for 90 min at 37°C using Liberase Blendzyme 2 (Roche), DNase I, and *Nα-p*-tosyl-L-lysine chloromethyl ketone (Sigma-Aldrich) in calcium-containing HBSS. Brain suspensions were centrifuged, resuspended in 30% Percoll, and overlaid onto 70% Percoll. Cells were harvested from the interface and counted. Cervical LNs collected included the submandibular/superficial, jugular/facial, and deep. Cells were Fc blocked in FACS buffer and incubated with respective Abs or isotype controls for 20 min at 4°C. Cells were washed and analyzed using a FACSCalibur and CellQuest Pro (BD Biosciences) or FlowJo (Tree Star) software.

Results and Discussion

To define the mechanism by which CD8$^+$ T cells may encounter IC Ag, we used cells from mice which express membrane-bound OVA but are deficient in MHC class I H-2K^b (mOVA/K$^{b-/-}$), ensuring that the transferred Ag cannot directly engage host T cells and therefore must be cross-presented. As a readout

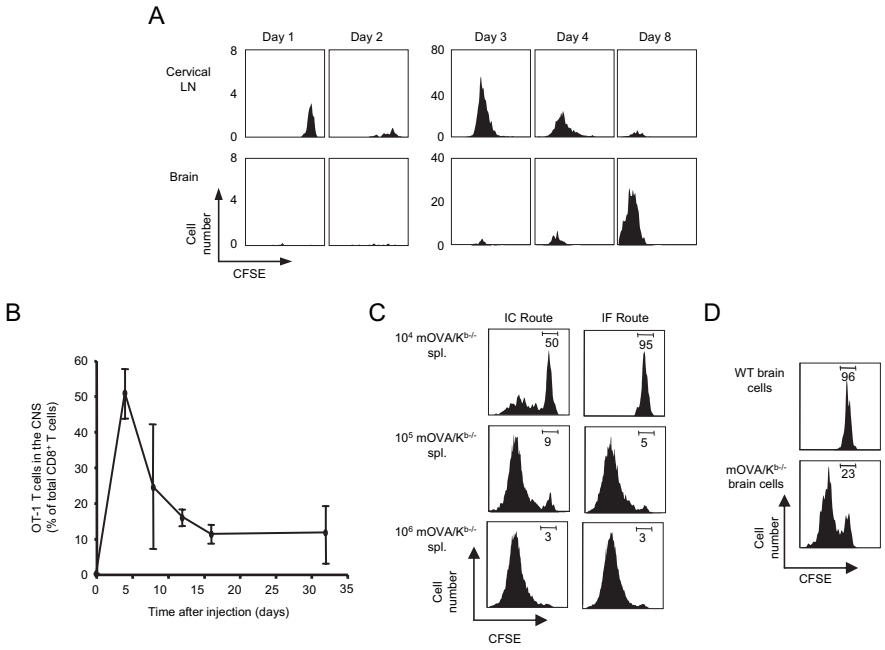

FIGURE 3. Following cross-presentation of IC Ag, CD8$^+$ T cells undergo divisions in draining cervical LNs and return to the site of Ag administration. *A–D,* CFSE-labeled OT-1 cells were adoptively transferred into WT mice, and mOVA/K$^{b-/-}$ splenocytes were injected via the IC route. *A,* Cervical LNs and brain cerebrums were isolated and processed for flow cytometry analysis at the indicated time points. OT-1 cells were gated based on their expression of CD45.1 and CD8. Data are representative of $n = 2$–3 mice/condition. *B,* The number of OT-1 cells within the brain that had divided at least once is shown in mice after IC immunization. Data are mean values ± SD from duplicate mice and are representative of two experiments. *C,* OT-1 proliferation is shown from the cervical LNs of mice that received IC brain cells from either WT or mOVA/K$^{b-/-}$. The percentage of undivided cells is indicated. Data are representative of four mice per condition. *D,* WT mice were injected with increasing numbers of mOVA/K$^{b-/-}$ splenocytes. The percentage of undivided cells is indicated. Data are representative of four to six mice per condition performed in two experiments.

of effector CD8$^+$ T cell activity, we monitored in vivo cytotoxicity and ex vivo IFN-γ production in CD8$^+$ splenocytes. Recipient WT mice received increasing numbers of mOVA/K$^{b-/-}$ splenocytes IC (Fig. 1*A*). DCs (10^5) loaded with titrated concentrations of OVAp were used as a positive control (Fig. 1*B*). Cross-presented IC Ag induced a robust in vivo CTL response (Fig. 1*A*) and stimulated an increased precursor frequency of Ag-specific IFN-γ-producing cells (Fig. 1*C*). These data demonstrate that cross-presentation of 10^5 splenocytes is as efficient as 10^5 DCs pulsed with 10 nM OVAp. As expected, killing responses were dependent on the amount of Ag injected, but to our surprise, cross-priming via the IC route was ~10-fold more efficient than the IF route (Fig. 1*A*). Control experiments confirmed that splenocytes were not leaking into the i.v. circulation after IC injection (data not shown). In addition, efficient CTL cross-priming after IC injection was observed when using non-migratory mouse embryonic fibroblasts derived from mOVA/K$^{b-/-}$ mice (data not shown). Given the lack of conventional lymphatic pathways, it was important to determine where the priming was occurring and why the IC route is so efficient.

To characterize which LNs harbor Ag following IC injection, fluorescent beads were injected into the right cerebral hemisphere. On days 1 and 2 postinjection, leukocytes were isolated from regional LNs, distal LNs, the spleen, and both cerebral hemispheres. Cells were labeled for cell surface markers to allow for discrimination of bead-positive DCs (Fig. 2*A*). Bead-positive cells were detected in the submandibular and deep cervical LNs, 10–20% of which were DCs (Fig. 2*A* and data not shown). At this time, we also observed bead-positive macrophages, indicating that beads may have directly trafficked to the cervical LN. Notably, between days 1 and 2, we observed a 2-fold increase in the number of bead-positive DCs recovered from the submandibular LNs, suggesting that many of the beads had been transported there as cell-associated particles (data not shown). Moreover, the number of bead-positive cells in the jugular LNs and distal LNs were below the level of detection (Fig. 2*B*), indicating that trafficking of beads from the cerebral areas is restricted to specific LNs. Both the contralateral and ipsilateral LNs showed similar numbers of bead-positive DCs (Fig. 2*B*), suggesting either bilateral drainage or Ag transfer from the right to left cerebral hemispheres during IC injection due to the small size and inert nature of the particles.

To examine the utilization of the submandibular LNs after i.v. injection of Ag, beads were administered i.v. Under these conditions, beads were primarily found within the spleen (Fig. 2*C*). Following IC administration of beads, >90% of bead-positive cells were CD11b$^+$/CD11c$^-$ macrophages/microglia (data not shown). Interestingly, we did observe an influx of host DCs to the injection site and, strikingly, >40% of the DCs were bead positive (Fig. 2*D*). Although the presence of bead-positive DCs in the CNS could account for the specific trafficking of Ag to the submandibular LNs, we could not exclude the possibility that even with a 2-μm diameter, the beads could traffic as free particles to the LN and subsequently be captured by lymphatic DCs.

To validate these findings using cell-associated Ag, we next injected mOVA/K$^{b-/-}$ splenocytes via the IC route. OT-1 were used as the responder cells following IC injection. The OT-1 cells were labeled with CFSE and adoptively transferred

into WT recipients. On day 2, it was possible to observe the first cell divisions in the submandibular LNs and only on day 4 was it possible to detect divided T cells in the brain (Fig. 3*A*). Interestingly, the OT-1 population made up a substantial number of brain $CD8^+$ leukocytes and persisted for >30 days (Fig. 3*B*). Similar results were found with IC injection of OVAp-loaded DCs (data not shown). As an additional set of control experiments, we ruled out the possibility that IC-injected cells were exiting the brain via the injection site itself and trafficking to the submandibular LN via skin afferent lymphatics (data not shown). Finally, we injected brain cells prepared from mOVA/$K^{b-/-}$ mice and administered them via the IC route into WT mice. Again, we were able to detect OT-1 T cell proliferation in the cervical LN, indicating that Ags derived from nonmigratory cells may be cross-presented by host APCs (Fig. 3*C*). These data support the conclusion that cross-priming of IC Ag occurs in regional LNs, after which primed T cells are able to enter the CNS.

We next compared the efficiency of cross-presentation after injecting mOVA/$K^{b-/-}$ cells via the IC or IF route. A more robust proliferative response was observed following Ag cross-presentation from the IC route compared with the IF route at all concentrations examined (Fig. 3*D*). We believe that this accounts for the stronger priming of the endogenous repertoire as shown above (Fig. 1). Within the CNS, DCs remain the best candidate for the APC responsible for the capture and cross-presentation of brain Ag (4, 6, 15). As our experimental model explored only situations of brain inflammation secondary to the injection of Ag, both resident and infiltrating DCs are candidates for the responsible APC (8, 16, 17).

We next determined the cellular requirements for in vivo cross-priming. Mice were depleted of $CD4^+$ T cells before immunization and, as seen in other models, we observed a complete dependence on the presence of $CD4^+$ helper T cells (Fig. 4*A*). In contrast, direct priming remained intact (Fig. 4*A*). Since several studies have demonstrated that $CD4^+$ T cell help is provided through CD40 signaling (18, 19), we investigated whether the same is true for cross-priming via the IC route. WT and $CD40^{-/-}$ mice were primed using DC/OVAp or mOVA/$K^{b-/-}$ splenocytes to assess the requirement for CD40 signaling in direct vs cross-priming of $CD8^+$ T cells, respectively. Although the in vivo killing remained intact in $CD40^{-/-}$ mice for the direct priming of $CD8^+$ T cells, the cross-priming response was completely inhibited (Fig. 4*B*). Our previous studies have shown that $CD4^+$ T cell licensing of DCs results in the secretion of IL-12 and IL-6 (20); therefore, using available knockout animals, we conducted a comparison of direct vs cross-priming in IL-$12p35^{-/-}$ and IL-$6^{-/-}$ mice. We found that neither IL-12p35 nor IL-6 is critical for the priming efficiency of $CD8^+$ T cells following IC injection (Fig. 4*B*), suggesting that there may be several downstream molecules involved in the CD40-mediated helper effect. Together, the data in Fig. 4 indicate that $CD4^+$ T cell help is required for cross-priming brain Ag-specific $CD8^+$ T cells and that this licensing event is dependent on CD40 signaling.

Our results demonstrate that following the injection of cell-associated Ag into the IC compartment, expansion and $CD8^+$ T cell differentiation occur in the LNs and not within the CNS itself. Only after T cell activation can responding $CD8^+$ T cells enter the brain. Although these observations are supported by several studies that investigated priming of Ag-specific T cells

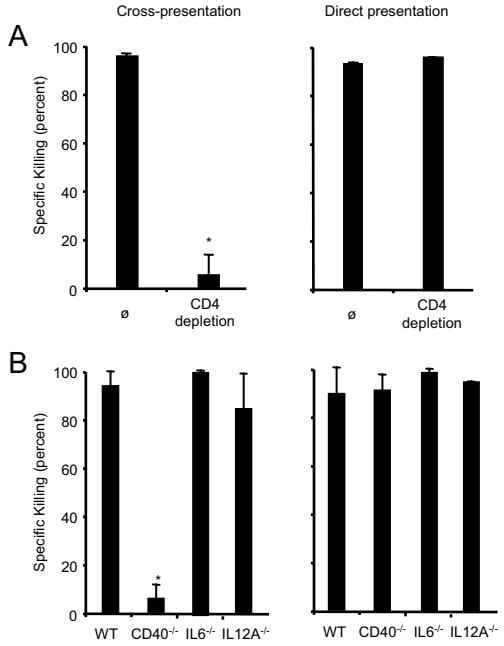

FIGURE 4. Efficient cross-priming of $CD8^+$ T cells by IC Ag requires $CD4^+$ T cell help and CD40 expression. *A,* WT mice were depleted of $CD4^+$ cells by i.p. administration of anti-CD4 mAb before IC administration of mOVA/$K^{b-/-}$ splenocytes or DC/OVAp. An in vivo killing assay was performed. Data are mean values + SD from $n = 4–6$ mice/condition; *, Student's t test, $p < 0.05$, statistically significant difference between WT and CD4-depleted mice for in vivo killing responses following cross-presentation. *B,* In vivo killing assay was performed in WT, $CD40^{-/-}$, IL-$6^{-/-}$, and IL-$12p35^{-/-}$ mice after IC injection of mOVA/$K^{b-/-}$ splenocytes or DCs/OVAp. Data are pooled from two to three experiments and Ag-specific killing was averaged. Mean values +SD from $n = 4–8$ mice/condition are shown; *, Student's t test, $p < 0.05$.

following IC injection of Ag (8, 11, 21), our findings differ from the recent study by McMahon et al. (6). Using a model of $CD4^+$ T cell-mediated experimental autoimmune encephalomyelitis, they demonstrated that T cell priming occurs within the CNS. One possibility is that the discrepancy is due to differential requirements for the priming of $CD4^+$ vs $CD8^+$ T cells, but may also reflect the choice of experimental system and in particular the form or nature of the Ag. Although it is not surprising that cross-presentation resulted in the priming of $CD8^+$ T cells following brain inflammation (22), it is unclear whether a constitutive pathway for cross-tolerance exists (23). Alternatively, in immune-mediated CNS disease pathogenesis, the expression of "CNS-specific" Ags outside the CNS may contribute to the initial activation of Ag-specific $CD8^+$ T cells that once activated would be capable of entering the brain and initiating disease, in turn triggering a feed-forward response.

In summary, our data offer mechanistic information regarding the cross-priming of $CD8^+$ T cells in vivo due to cross-presentation of IC Ag. We also show that the initial priming event of $CD8^+$ T cells must occur in the cervical LNs before $CD8^+$ T cells entering the CNS. Trafficking of IC Ag following brain injury is highly immunogenic; nevertheless, cross-priming is dependent on $CD4^+$ T cell help. Based on the failure to cross-prime T cells in $CD40^{-/-}$ mice, we believe this helper response acts via a "licensing" event that is similar to what has

been shown for other cell-restricted Ags (18, 24). This experimental system will permit the further characterization of effector mechanisms relevant to the pathogenesis of CNS immune disorders.

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Disclosures

The authors have no financial conflict of interest.

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