SUPPLEMENTAL MATERIAL

Hao et al., http://www.jem.org/cgi/content/full/jem.20092749/DC1

Intracranial cannula implantation. Mice were anesthetized with 87 mg/kg ketamine and 13 mg/kg xylazine and placed in the stereotactic apparatus (Kopf Instruments). A sagittal incision ~2.0 cm in length was made, the skin was retracted, and the skull was exposed and cleaned. Unilateral cannulae (Plastic One) connected to Alzet Osmotic minipumps (model 1002) were aimed at the intracerebellomedullary cistern with the incisor bar and the cannulae angled 10° toward the midline of the brain. The cannulae were secured to the skull with contact glue and dental cement. Once the cement solidified, an incision was made in the intrascapular space to insert the minipump. The incision was then sutured, and the mice were allowed to recover for a period of 3 d before infusion began. The correct placement of cannulae was determined by histological examination. Brain sections were stained with cresyl violet, and the tips of the cannulae were located. Mice in which the cannulae were found to be misplaced were excluded from the experiment. To deplete NK cells, anti-NK1.1 antibody was administered via the implanted cannula at 200 μg intracerebral at days −2, 5, 9, 12, 20, and 25, after immunization.

Cytokine array. The overall cytokine levels in CNS tissue homogenates and lymph node culture supernatants were measured by the Multi-Analyte ELISArray kit (SA Biosciences). Protein homogenates were extracted from the mouse brain using the Halt Protease Inhibitor Cocktail kit (Thermo Fisher Scientific), centrifuged at 78,500 *g* for 1 h at 4°C, and the supernatants were collected and stored at −70°C until assayed. According to the kit's manual instructions, the levels of 12 cytokines and chemokines (IL-1β, IL-4, IL-6, IL-10, IL-12, IL-17a, IFN-γ, TNF, TGF-β1, MCP-1, MIP-1α, and MIP-1β) were detected simultaneously at 1:20 dilutions. Negative and positive controls supplied by the kits were also included. The reaction was analyzed at a wavelength of 450 nm using a 96-well microplate reader (Model 680; Bio-Rad Laboratories).

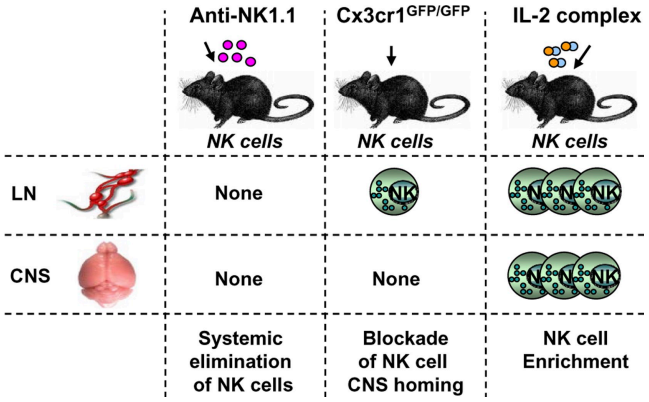

Figure S1. Approaches to study NK cells during CNS inflammation. Independent and complementary approaches were used. The first approach consists of depleting NK cells using a monoclonal antibody, derived from clone PK136, directed against the NK cell marker NK1.1. Systemic NK cell depletion permits us to asses the impact of NK cells during different stages of EAE development and to compare the impact of NK cell depletion on Th17 cells in the periphery and CNS. The second approach employs CX3CR1-deficient mice on a B6 background. Homing of NK cells to the CNS, but not to other compartments, is severely compromised in these animals. This approach allows us to asses the impact of NK cells on Th17 cells within the CNS. The third approach involves NK cell expansion via engagement of the IL-2 receptor via IL-2–IL-2 mAb (S4B6 clone) complexes. This technique should allow us to confirm our findings obtained in NK-deficient models and to explore the feasibility of targeting NK cells for therapeutic purposes.

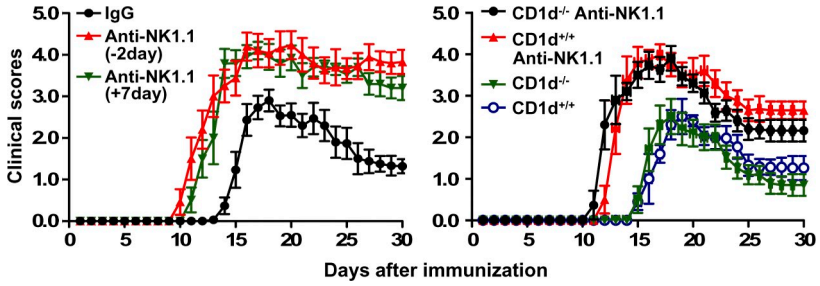

Figure S2. Effect of anti-NK1.1 mAb on the development and progression of EAE in B6 mice and in CD1d$^{-/-}$ mice. NK1.1^{+} cell depletion was achieved by treating mice with anti-NK1.1 mAb. Mouse IgG was used as control Ab. (Right) Clinical EAE in B6 mice that received anti-NK1.1 mAb starting at days −2 or +7 after immunization with MOG_{35-55}/CFA/PT (n = 25–35/group). The data are pooled from three similar experiments. For comparisons between mice that received anti-NK1.1 and IgG, Error bars represent the means ± SEM. $P < 0.05$ between days 12 and 24 after immunization. $P < 0.01$ after day 17 after immunization. For comparisons between mice that received anti-NK1.1 at day −2 or +7, $P > 0.05$ for all time points. P-values were determined by the Mann-Whitney U test. (Left) Development of EAE and effect of anti-NK1.1 mAb in CD1d$^{-/-}$ mice (crossed to B6 mice for 11 generations) devoid of NKT cells (n = 12–15/group). For comparisons between mice that received anti-NK1.1 or IgG, $P < 0.01$ after day 16 after immunization. $P > 0.05$ for any other comparisons. Data are pooled from three similar experiments (Mann-Whitney U test). Error bars represent the means ± SEM.

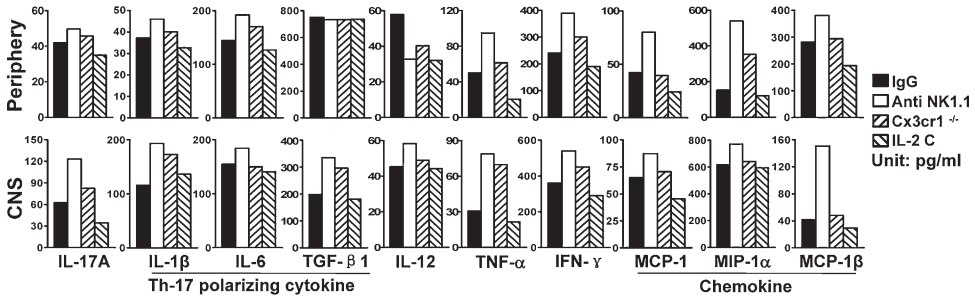

Figure S3. Global alteration of Th17-polarizing cytokines and other inflammatory mediators in mice when NK cells are manipulated. Lymph node culture supernatants and CNS homogenates were prepared from mice of the indicated groups at day 15 after immunization. Cytokine concentrations were measured by a Multi-Analyte ELISArray kit (SA Biosciences). n = 4/group.

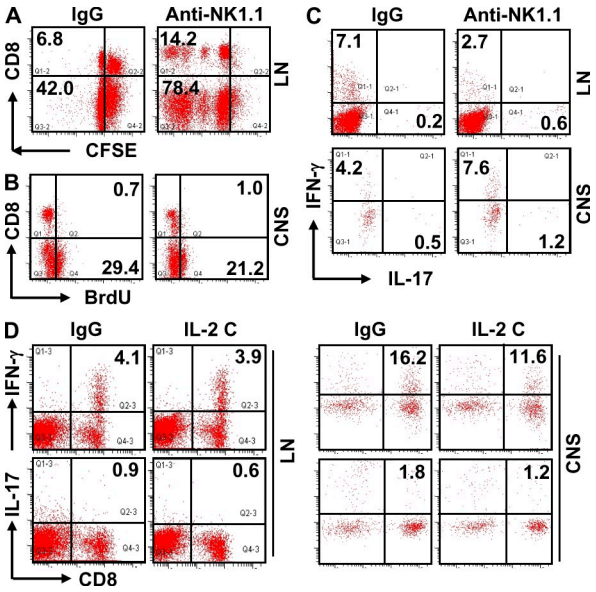

Figure S4. Effects of anti-NK1.1 mAb or IL-2 C injection on CD8⁺ T cell responses. Groups of mice (receiving control or anti-NK1.1 mAb or IL-2 C) were immunized with MOG/CFA/PT. These mice were sacrificed between days 12 and 20 after immunization, and lymph node and CNS cells were isolated. (A) Expansion of lymphoid CD8⁺ T cells was assessed by CFSE dilution. (B) Expansion of CD8⁺ T cells in the CNS was assessed by BrdU incorporation assay. (C) Lymphoid or CNS cells were restimulated with MOG overnight, and IFN-γ– and IL-17–expressing CD8⁺ T cells were measured by intracellular staining. IFN-γ and IL-17 production by CD8⁺ cells from lymph node or the CNS was analyzed by FACS. (D) Effect of IL-2 C on myelin-reactive, IFN-γ–, and IL-17–producing CD8⁺ cells in the periphery and the CNS. The data for A–D shown is from one experiment of three with each individual observation containing pooled cells from 20–25 mice. Dot plots were generated after gating on lymphocytes (by forward vs. side scatter).

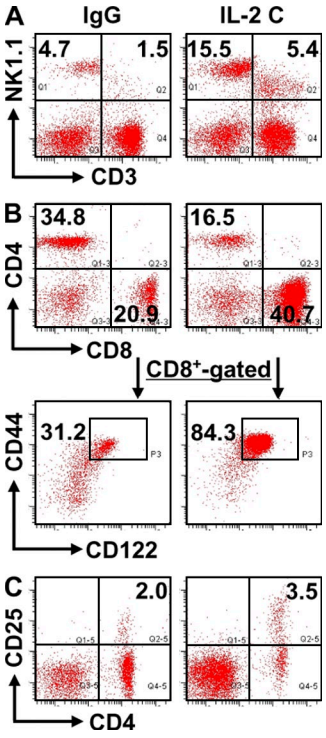

Figure S5. Effects of IL-2 immune complexes on NK (CD3⁻NK1.1⁺), NKT (CD3⁺NK1.1⁺), CD4⁺, CD4⁺CD25⁺, and CD4⁺CD25⁺T cells. Upon EAE induction with MOG, B6 mice were treated with 1.5 µg IL-2/mouse plus 15 µg of anti–IL-2 mAb/mouse or IgG i.p. daily for a total of seven doses. Single cell suspensions were isolated from the spleen and CNS of mice on days 12–20 after immunization. The dot plots are representative of three separate experiments (n = 12–15/group).

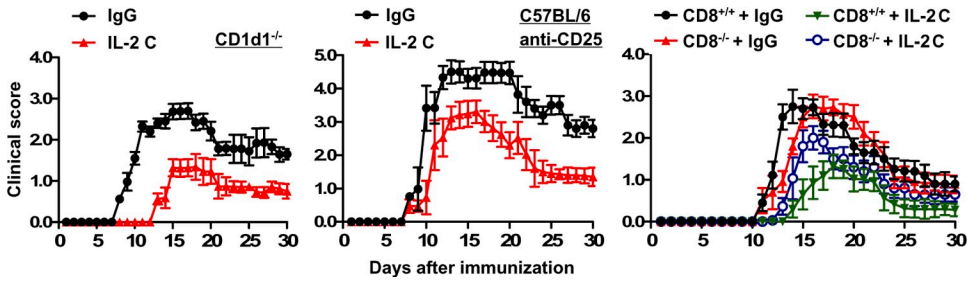

Figure S6. Contributions of IL-2 receptor bearing NKT cells, T reg cells, and CD8⁺ cells to protection against EAE conferred by IL-2 complexes. Groups of WT, CD1d$^{-/-}$, or CD8$^{-/-}$ mice were primed with MOG/CFA, and IL-2 complexes were administered upon EAE induction. In separate experiments, mice received anti-CD25 and IL-2 complexes simultaneously upon EAE induction. n = 6–11/group. For CD1d$^{-/-}$ mice, $P < 0.01$ between days 9 and 21 after immunization and $P < 0.05$ after day 22 after immunization. For mice that received anti-CD25 mAb, $P < 0.05$ after day 13 after immunization. For CD8$^{-/-}$ mice, $P = 0.058$ between groups of CD8$^{-/-}$ + IgG and CD8$^{-/-}$ + IL-2 C between days 10 and 23 after immunization. $P < 0.05$ for the remaining time points. Data are pooled from two to three similar experiments. Error bars represent the means ± SEM. P-values were determined by the Mann-Whitney *U* test.

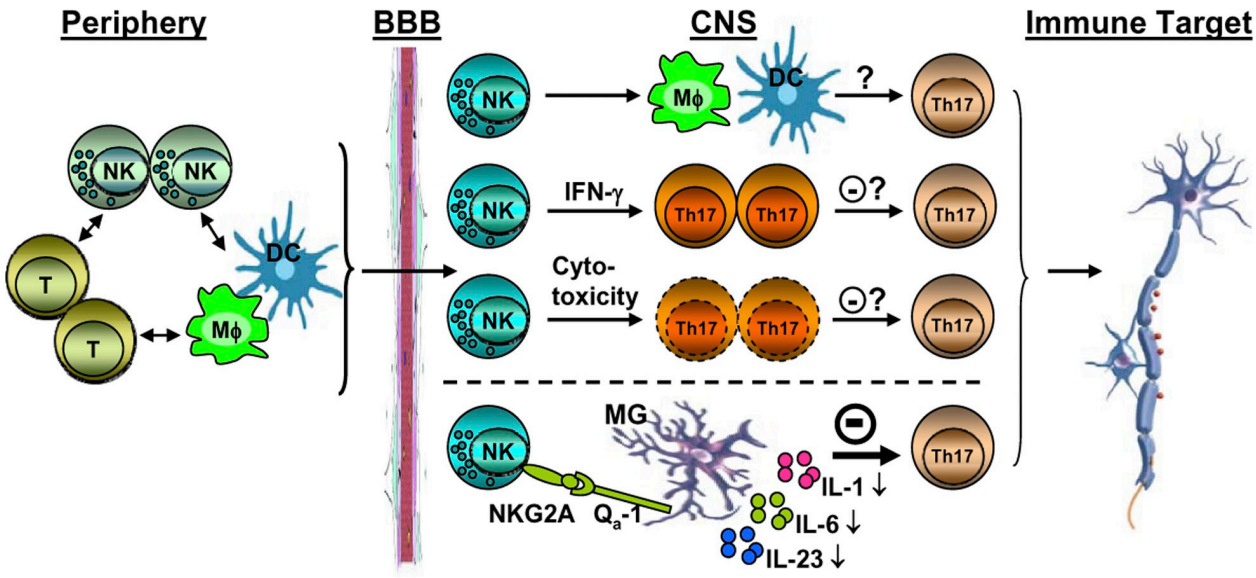

Figure S7. NK cell–mediated inhibition of Th17 cells and autoimmunity in the CNS. During the initiation and progression of CNS inflammation/autoimmunity, as reflected in EAE or MS, NK cells, DCs, and macrophages, together with peripherally activated myelin-reactive T cells, transmigrate into the CNS. Before the arrival of these cells into the CNS, CNS-resident APCs, such as microglia, are already activated and proliferate. APCs recruited from the periphery and microglia may present endogenous CNS myelin antigens to previously activated T helper cells. Microglia, in the absence of suppressive action from NK cells in situ, fuel the process via swift proliferation with release of abundant Th17-polarizing cytokines. Consequently, myelin-reactive CD4⁺ T cells expand and further differentiate. Together with other cells and inflammatory mediators, CD4⁺ T cells coordinate damaging events in the CNS. The nature of the interaction between NK cells and microglia involves cytolysis, predominantly mediated by the NKG2A–Qa1 pathway. This interaction significantly shapes autoreactive Th17 cell responses and subsequent CNS pathology. The possibility that NK cell–derived IFN-γ plays a role, or that direct killing of T cells contributes to the inhibitory action of NK cells within the CNS, is also depicted, although evidence supporting these mechanisms is less convincing or yet to be established in our model. BBB, blood brain barrier.

Table S1. Effects of IL-2 complexes on induction and maintenance of NK cells, as well as clinical features of EAE

Treatment regimen	Maintenance of NK number	Duration of maintenance of NK number	Delay of EAE onset	Clinical feature of EAE
	×10⁶	*×10⁶*	*day after immunization*	
7 d consecutive then stop	6.3± 1.5	8.2 ± 2.1	7.5± 1.3	Loss of protection in about 5 d, mean clinical score indistinguishable with IgG-treated mice by day 18 p.i.
Two injections per week throughout	5.2 ± 1.1	Maintain throughout Exp	6.5 ± 2.0	Maintain partial protection
Four injections per week throughout	6.0 ± 1.4	Maintain throughout Exp	6.2 ± 1.7	Maintain disease protection similar to three injections per week

To optimize the IL-2 complex treatment regimen, three protocols were compared: (1) 7 d of consecutive injection only did not maintain NK cells on a long-term basis or delay EAE onset, and mice lost protection by day 18 after immunization; (2) two injections per week throughout maintained NK cells but conferred only partial disease protection; (3) three or four injections per week throughout maintained NK cells and conferred disease protection. Thus, three injections per week was chosen as the standard protocol.