Mycobacterium smegmatis Expressing a Chimeric Protein MPT64-Proteolipid Protein (PLP) 139–151 Reorganizes the PLP-Specific T Cell Repertoire Favoring a CD8-Mediated Response and Induces a Relapsing Experimental Autoimmune Encephalomyelitis

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Mycobacterium smegmatis Expressing a Chimeric Protein MPT64-Proteolipid Protein (PLP) 139–151 Reorganizes the PLP-Specific T Cell Repertoire Favoring a CD8-Mediated Response and Induces a Relapsing Experimental Autoimmune Encephalomyelitis

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We infected SJL mice with a recombinant Mycobacterium smegmatis expressing a chimeric protein containing the self-epitope of proteolipid protein 139–151 (p139) fused to MPT64, a secreted protein of Mycobacterium tuberculosis (rMSp139). Infected mice developed a relapsing experimental autoimmune encephalomyelitis (EAE), showing a prevailing demyelination of the CNS, and disease severity was significantly lower in comparison with the one that follows immunization with p139. rMSp139 was not detected in lymph node or spleen in the course of clinical disease development or in the CNS during relapse. Infection with rMSp139 modified the p139-specific T cell repertoire, recruiting the spontaneous p139-specific repertoire and activating CD4+ T cells carrying the BV4 semiprivate rearrangement. T cells carrying the public BV10 rearrangement that are consistently found in lymph node or spleen in the course of clinical disease development or in the CNS during relapse. Infection with rMSp139 selectively fail to present the epitope for which BV10 cells are specific. Simultaneously, rMSp139 expanded p139-specific CD8+ cells more efficiently than immunization with peptide in adjuvant. SJL mice vaccinated against the CDR3 sequence of the BV10 public protein; MEF, mouse embryonic fibroblast; MS, multiple sclerosis; NTS, nuclear target sequence; p139, proteolipid protein peptide 139–151; PLP, proteolipid protein; R-EAE, relapsing experimental autoimmune encephalomyelitis; RSI, relative stimulation index.

A widely accepted hypothesis suggests that infectious agents mimicking self-Ags induce autoimmune diseases by priming self-reactive T cells in the periphery. These T cells migrate into the target organ and contribute to tissue damage. This postulate was elegantly demonstrated by Ohashi et al. (1) and by Oldstone et al. (2) where they showed that infection with viruses was able to trigger the development of diabetes in mice expressing viral proteins under the control of the insulin promoter. A role for self-mimicry in experimental autoimmune encephalomyelitis (EAE) is supported by the observation that intracerebral infection of SJL mice with Theiler’s virus encoding for myelin peptide PLP 139–151 (p139) results in acceleration of the encephalomyelitis mediated by proteolipid protein (PLP)-specific T cells (3). Treatment of multiple sclerosis (MS) patients with an anti–VLA-4 mAb decreases disease relapses (4), supporting the hypothesis that activated encephalitogenic T cells in MS come from peripheral lymphoid organs. Also, microbial peptides presented by DR2-encoded molecules activate a T cell specific for myelin basic protein (MBP) (although with a lower avidity than the self-MBP peptide) and induce EAE in a transgenic mouse model (5). As yet, there is no good experimental model that reproduces the pathology of MS upon infection with a living agent, priming of self-reactive T cells, elicitation of an autoimmune disease without the presence of the infectious agent in the target organ.

At least three steps are critical for the development of autoimmune diseases. First, an appropriate T cell repertoire has to be activated. In fact, activated self-reactive T cells of healthy and sick individuals belong to distinct repertoires in both experimental and human autoimmune diseases (6, 7). Second, polarization of T cells toward a pathogenic phenotype needs to be induced. Polarization toward pathogenic, Th1, and Th17 phenotypes depends on the cytokine milieu in which priming occurs, and this

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in turn is regulated by the engagement of pathogen-associated molecular pattern receptors on a dendritic cell (DC). Third, pathogenic T cells need to home into the target organ. In addition to polarization, the environment in which priming occurs also influences the trafficking properties of T cells (8), possibly through regulation of integrin expression by Ag-presenting DCs (9, 10).

Because of the peculiar functional characteristics of the blood-brain barrier (BBB), homing to the CNS is a step that possibly limits severely the development of MS and of its experimental counterpart, thereby balancing the failure of Ags restricted to the CNS to induce peripheral and central tolerance (11, 12). CNS infection can alter the BBB overcoming this “check point,” and several observations suggest that CNS infections by viruses and other pathogens are likely to be involved in MS pathogenesis (13), associated with or instead of peripheral infections by cross-reactive agents (14). In the SJL mouse, EAE follows immunization with p139 alone without administration of pertussis toxin, indicating that p139-specific T cells primed in the periphery acquire a phenotype sufficient for homing into the CNS without need for alteration of the BBB. The development of EAE is critically dependent on the presence and amount of Mycobacterium tuberculosis products in the adjuvant (15) that provide nonantigenic moieties, thereby determining the encephalitogenic phenotype during priming. We therefore wondered whether infection with nonpathogenic mycobacteria carrying a determinant cross-reactive toire, obtained by means of vaccination against the sequence of the BV-BJ spectratyping, the so-called immunoscope) shows differences in the repertoire of CD4+ and CD8+ T cells recruited by infection with live M. smegmatis compared with immunization with p139 in adjuvant. A comparable modification of the CD4+ repertoire, obtained by means of vaccination against the sequence of the public BV10-B11.1 of 97b length (herewith BV10) CDR3, results in reduction of EAE symptoms during the acute bout of disease. Thus, a transient infection with CNS-cross-reactive mycobacteria induces an R-EAE after the infectious agent has been cleared. The composition of the self-reactive repertoire activated following infection can determine the severity of the resulting disease.

**Materials and Methods**

**Mice, peptide, and immunization**

Female SJL mice (2 mo old; Charles River Laboratories, Calco, Italy) were used in the experiments reported. p139 (Ser140) was purchased from PRMM (Milan, Italy) and was >95% pure, as determined by HPLC and mass spectroscopy.

Mice were immunized s.c. with 50 μg/mouse of p139 in PBS, emulsified 1:1 with CFA (CFA is immunofluorescence assay [IFA] containing 1 mg/ml of killed and heat dried M. tuberculosis H37RA) or emulsified in IFA containing 4 mg/ml killed and heat-dried M. tuberculosis H37RA (enriched CFA) (Sigma-Aldrich, St. Louis, MO) in a final volume of 100 μl/mouse. All experimental procedures involving animals were approved by the internal Ethical Committee.

**Construction of plasmids and recombinant M. smegmatis**

The mpt64 gene expressed in this work was amplified with Pfu DNA polymerase (Invitrogen, San Diego, CA) using the H37Rv genome as template. The forward primer (64Pr5Kpn, 5'-ACCCGATACCCCTGA GCACCCAGCGCAACCCGGCC-3') was designed to contain a KpnI site immediately before the putative promoter of the mpt64-coding sequence, whereas the reverse primer (64wh3Xb, 5'-AACCCTGAGCCGCA CGTACACCCGGAAACC-3') was designed to have an XbaI site before the stop codon. For the p139 (Ser140) epitope, the forward primer was designed to contain a p139-coding sequence in frame to the coding sequence of the gene of interest and an XbaI site, whereas the reverse primer was designed to contain the stop codon and a BamHI site. The PCR product was cloned initially into the Zero Blunt cloning vector (Invitrogen) and then inserted into the mycobacterial expression vectors pMV206 digested with KpnI and BamHI. A control plasmid was also produced that encoded the sequence Y P Y D V P D Y A S L (residues 114–124 of the precursor of hemagglutinin A [HA]), encompassing the epitope that is immunodominant for Ab response.

To evaluate in vivo expression of the mpt64 gene following infection, mice were infected with 107 CFU/mice of mpt64. At different time points mice were sacrificed for the determination of bacterial loads and extraction of total RNA. Lymph nodes (LNs) and spleens were homogenized in PBS containing 0.02% Tween 80, and serial dilutions were plated onto Middlebrook 7H11 agar (Difco). Colonies were counted after 1 wk of incubation at 37°C. In vivo expression of mpt64 in spleen and LN are provided as a ratio compared with the expression of the 16S rRNA gene by real-time RT-PCR using an ABI 7000 (Applied Biosystems, Foster City, CA) (27). The following primers were used: 16S rRNA, primer F, 5’-CAAGGCTTTCACATGCTCATA-3’; primer R, 5’-GATTCACGAGCAGTTTCCT-3’; and probe, Vic (5’-TCTGGTATCTACGAC TCGACTTCCAG-3’-TAMRA) and MPT64, primer F (5’-GGCGGCC GAGATCCAATGTT-3’), primer R (5’-GGCGGCAGCTGATGTTG-3’), and probe, Fam (5’-CGACGCCGCTTACA-3’-TAMRA).

Data were analyzed by one-way ANOVA, and significant differences between the means were measured by using Tukey’s test. A value of p < 0.001 was considered significant.

**EAE induction and extraction of infiltrating cells from the CNS**

Mice were immunized s.c. in the back with 75 μg/mouse of p139 emulsified in enriched CFA (100 μl/mouse), or infected with doses of rMS ranging from 104 to 4 × 107 CFU or with 4 × 106 CFU of M. smegmatis. Mice were scored for clinical signs of disease according to the following scale: 0, no clinical score; 1, loss of tail tonicity; 2, weak hind leg paresis; 3, posterior leg paresis; 4, complete paraplegia; and 5, death. Intermediate values were given for incomplete symptoms. Mice were scored by two independent observers with difference being within 0.25 points; thus, the average scores are reported.

CNS was collected from mice and reduced to a pulp by crush (mashed). Percoll gradient was performed, and mononuclear cells were extracted at the 30–70% interface. Collected cells were washed twice with 10× 106 αβ-BW cells (as previously described for analysis of low numbers of T cells [28] and resuspended in RL buffer for RNA extraction.

**CNS histology**

Mice infected with rMS or immunized with p139 were sacrificed and perfused through the aorta with 50 ml of saline solution, followed by 50 ml of 0.01 M (pH 7.4) PBS and 4% paraformaldehyde under deep anesthesia (ketamine/diazepam 1:1 i.p.). Brains and spinal cords were removed and immersed in the same fixative for 24 h. Tissue blocks were routinely embedded in paraffin. Serial 10-μm coronal sections of the brain and serial 10-μm transverse or longitudinal sections of the spinal cord were cut on a microtome and then processed for histological analysis (H&E for routine examination or Wolcke for myelin staining) or immunocytochemistry to
reveal inflammatory cells (CD3-expressing lymphocytes and activated microglia). For CD3 labeling, sections were incubated overnight at +4˚C with the primary Ab (rat monoclonal anti-CD3; BD Biosciences, Franklin Lakes, NJ) and then for 1 h with a biotin-conjugated secondary Ab (Vector Laboratories, Burlingame, CA). Microglia cells were identified with the specific histochemical marker Ricianus Communis Agglutinin I (bionylated Ricianus Communis Agglutinin 120; Vector Laboratories). Immunoreactive cells were visualized by the avidin-biotin immunoperoxide method with diaminobenzidine as chromogen (Vectorstain Elite ABC Kit; Vector Laboratories).

Ex vivo APC population enrichment

Four 8 wk old SJL female mice were infected with 10³ CFU of M* smegmatis i.p. Four days later, DC populations were enriched from draining LNs following the protocol described in Ref. 29. Briefly, LN cells (LNCs) depleted of T cells were separated in low- and high-buoyant density cells by centrifugation over a discontinuous Percoll (Phamacia LKB, Uppsala, Sweden) gradient containing a 55% layer. Low-buoyant cells were separated by MiniMACS separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany) using B220-specific microbeads. The APC population enrichment was checked by analysis on FACScan flow cytometer (BD Biosciences, Mountain View, CA) equipped with Lysis II software.

Mouse fibroblast preparation and Ag pulsing

Mouse embryonic fibroblasts (MEFs) were prepared following the protocol described by Bryja et al. (30). MEFs were isolated from embryonic tissues obtained from SJL female mice 13 d postcoitum. Trypsinized embryonic tissues were cultured to generate MEF-1 cells, which were diluted 1/3, and allowed to grow to confluence to generate MEF-2 cells. MEF-2 cells were seeded at 5 x 10⁴ cells/mL in (24-well plate in 1 mL of complete medium; see below) in the presence or absence of 10 µg/ml p139. After 16 h, the supernatant was removed, and adherent cells were washed and layered with cells obtained from draining LNs of SJL mice that had been immunized with p139 in enriched CFA.

TCR repertoire analysis

Repertoire analysis was performed using a modification of a described protocol (31). A total of 5 x 10⁵ LNCs or 10⁴ spleen-derived cells/well were cultured in the presence or absence of 10 µg/ml p139 for 3 d in RPMI 1640 medium (Sigma-Aldrich) supplemented with 2 mM L-glutamine, 50 µM 2-ME, 50 µg/ml gentamicin (Sigma-Aldrich), and 10% FCS (Life Technologies, Basel, Switzerland) (complete medium). For the experiment reported in Fig. 3C, LNCs were incubated in the same medium without gentamicin with 10³ CFU of M* smegmatis. Gentamicin was added after 1 h, and 36 or 72 h later, cells were collected, washed in PBS, and resuspended in RLT. Total RNA was isolated from cell suspensions using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instruction. cDNA was synthesized using an oligo-dT primer (dT15) (Life Technologies). For complete “Immunoscope” analysis (28), cDNA was subjected to PCR amplification using a common Constant (C) β primer (CACT-GATGTTCTGTTGGTACA) in combination with the variable β (BV) pri-

CDSR3 sequencing

cDNAs were obtained from Ag-stimulated LNCs as described above. Each sample (2 µl) was submitted to a first PCR using the previously mentioned BV17-, BV20-, (for experiments reported in Supplemental Table II and Supplemental Table I), and BV10- (for the experiment reported in Fig. 5A and Supplemental Table II) specific forward primers and the common CB-specific reverse primer. A second nested PCR was then performed using 2 µl of the product of the former reaction as a template, the same BV-specific primer and BJ-specific reverse primers. PCR fragments were then cloned by using TOPO TA Cloning kit (Invitrogen), according to the manufacturer’s instructions. Transformed Escherichia coli were grown in 5 ml of Luria-Bertani medium supplemented with ampicillin, and plasmids were purified by Qiaprep Miniprep columns (Qiagen) and checked for the presence of the expected PCR amplification by cloning PCR products into M13 forward primers. Samples that scored positive for inserts were sequenced with a M13 forward primer using an Applied Biosystems 3130 Prism (Applied Biosystems). DNA sequence was translated into protein sequence through the ExPasy Proteomics Server (http://au.expasy.org/).

Anti-BV10 CDSR3-DNA vaccine preparation and intramuscular DNA electraference

The DNA vaccine specific for the BV10 p139-specific public TCR rearrangement was constructed in plasmid pRC110-NTS-IL-2 that was described previously (34). The nuclear targeting sequence (NTS) was used to facilitate nuclear plasmid uptake in murine skeletal muscle following naked DNA electroporation. Murine IL-2 was used as adjuvant. The plasmid vector was modified by a pair of complementary oligonucleotides, synthesized to assemble the double-stranded mini-fragments of the previously published BV10 CDSR3 “LYCASSPSGTNTETVFVGF” (28), using the following primers: sense, 5’-CTAGTAGAGCCACAAGTATGCCTTGCAACACCAGCCCCGGAAAAGCAGAGAGGTGATCTGATGAACTTGTCGTGGCTACACTAT-3’; and antisense, 5’-ATAGTTTGGGCGGCGTTAAAGCAAGAAGACTTCTGTTGTTGGCGGCGGT- CAGTGGACAGAGGATACTACGTGCCCGACTAGCAT-3’. Each primer incorporated a Nhel site (5’ end) and a Not site (3’ end), respectively (underlined sequence), to permit an oriented cloning. Single-strand oligonucleotides were in vitro phosphorylated and annealed by heating to 89˚C for 10 min and cooled down to 69˚C in 30 min and then to 65˚C in 10 min in 50 mM Tris-HCl, 10 mM MgCl₂, 10 mM DTT, 1 mM EDTA, 25 µg/ml BSA, 1× 10⁶ cells obtained from draining LNs with molecules with respective sticky restriction sites ends was checked by agarose gel electrophoresis. The obtained double-stranded molecule was inserted in pRC110-NTS-IL-2, and the resulting plasmid was named p139-specific T cells secreting IFN-γ, IL-4, and IL-10 were stained and enriched from LNs of SJL mice (immunized as described above) using MACS secretion kit (Miltenyi Biotec), according to the manufacturer’s instruction, following the protocols for enrichment of low-frequency secreting cells. Briefly, 1-3 x 10⁷ cells obtained using a flow cytometer were stimulated in the absence (background) or in the presence of 10 µg/ml p139 or the control peptide PLP178–191 in a 6-well plate at a concentration of 5 x 10⁴ cells/ml. Three hours later, cells were harvested and submitted to the staining procedure for each cytokine. The enrichment for cytokine-secreting cells was checked by flow cytometry (Coulter Epics FACS, equipped with Lysis software; Beckman Coulter, Fullerton, CA) analysis. To evaluate correctly the number of Ag-specific cells, we examined by FACS 5 x 10⁶ cells both in the background and positive samples. The number of Ag-specific, cytokine-secreting cells is obtained by subtracting the cells staining positively in the background sample from the number of the same cells in the Ag-stimulated sample. Total, negatively selected and positively selected cells were collected and prepped for mRNA isolation. To prevent uncontrolled loss of mRNA because of scarcity of cells, 10⁶ ρ of BW cells were added to the posi-

Staining and enrichment of IFN-γ, IL-4, and IL-10-secreting T cell

Gene-mapper version 4.0 software (Applied Biosystems). Results are also reported as RSI (relative stimulation index = normalized peak area obtained from cells stimulated with Ag + normalized peak area of non-stimulated cells). T cells carrying a TCR rearrangement were considered with the appropriate biotin-conjugated mAbs (BD Biosciences), followed by selection with streptavidin-conjugated MACS beads. A total of 2.5 x 10⁶ positively and negatively selected cells were cocultured in vitro with 10⁴ B220° cells selected from the spleens of naive SJL mice, in the presence of 10 µg/ml p139 for 3 d. Immunoscope analysis for T cells in each sample was performed as described above.
pBJCI10-NTS-IL-2-BV10. Plasmid DNA was purified using the Qiagen EpiPurePlasmid Mega Kit (Qiagen) and resuspended in sterile, endotoxin-free 150 mM sodium phosphate (pH 7.0). The CDR3 empty construct pRC110-NTS-IL-2 was used as control.

Mice were anesthetized by i.m. injection of a Ketamine-Domitor mixture in the muscle of the anterior limb according to the ethical committee for animal experiments guidelines. Then they were injected in both posterior muscle legs with plasmid solution (50 µg/50 µl) and subjected to electrotreatment with BTX ECM 830 Pulse Generator (Harvard Apparatus, San Diego, CA). A 175-V/cm voltage was applied in 10 pulses of 20-ms square wave pulses at 1 Hz. Muscles were pretreated with bovine hyaluronidase as reported elsewhere (35). Mice were treated twice, 3 wk apart. Two weeks after the second DNA electroporation, mice were immunized s.c. with 50 µg/mouse of p139 in PBS and emulsified 1:1 with CFA for immunoscope analysis (Fig. 5A) or with 75 µg/mouse of p139 emulsified in enriched CFA for the induction of EAE (Fig. 5B, 5C), as described above. The schedule of treatment that leads EAE induction resulted in a rate of mortality of 50%, irrespective of the plasmid used. Deaths occurred on day 1 or 2 postchallenge with p139. EAE score was monitored daily, blind with respect to vaccine treatment.

Results

Construction of the recombinant M. smegmatis expressing p139 and infection of SJL mice with the recombinant bacteria

MPT64 is an actively secreted, highly immunogenic Ag expressed by M. tuberculosis during the early stages of infection (36, 37). To express and present p139 (Seq456) in the context of a mycobacterial environment, the sequence encoding for this peptide was inserted downstream of the gene expressing the MPT64 protein in the mycobacterial shuttle-plasmid pMV206 (pMS60610). To ensure a high level of expression starting at the early stages of infection, the MPT64-P139 chimera was put under the control of the MPT64 promoter (38). M. smegmatis that does not naturally express MPT64 was transformed with the plasmid pMS60610, and expression of the chimeric protein is demonstrated by immunoblot analysis using an antisera specific for MPT64 (Fig. 4A).

SJL mice were infected s.c. with 106 CFU/animal of rMSp139, and bacterial persistence in draining LNs and spleen was assessed by CFU counting. As shown in Fig. 1B (filled symbols), CFU count in the LN was highest 2 h postinfection and declined thereafter, becoming undetectable by day 20 postinfection. A similar pattern was obtained for the spleen, where the highest bacterial burden was observed at day 3. These results are similar to what was observed in other previously published studies in B6 mice (39) and confirm that rMSp139 does not establish a stable infection also in SJL mice. A total of 106 CFU of rMSp139 correspond to 50 µg of rMSp139 dry weight, i.e., the same amount of heat-inactivated M. tuberculosis that is delivered to a mouse immunized with peptide in CFA.

The amount of mpt64-p139 transcript was determined during the time course of the infection, using quantitative real-time PCR of pooled LNs and spleens from the same mice up to day 10 postinfection (Fig. 1B, open symbols). Similar amounts of transcripts were observed in the LNs and spleen tissues despite a 10-fold difference in the number of CFUs present in these organs. DCs represent the main host for mycobacteria in LNs (40). However, they display a more hostile environment for the bacteria with respect to macrophages, which are possibly the main host in spleen (for a review, see Ref. 41). The difference in cell host possibly justifies the higher production of mRNA by rMSp139 in the spleen with respect to LNs. By extrapolating from the amount of mRNA detected in pooled lymphoid organs of infected mice (Fig. 1B), we calculated the total amount of p139 to which a mouse is exposed during the 10 d following infection with 106 CFU of rMSp139 to be on average 1.628 ± 0.014 ng, compared with the 50 µg/mouse of peptide used for immunization in adjuvant.

Infection with live rMSp139 induces an R-EAE

rMSp139 represents a model for nonpathogenic bacteria cross-reactive with a self-molecule. We therefore next determined whether infection with this live bacterium could actually induce clinical disease.

In a first experiment, three groups of SJL mice were immunized s.c. with 75 µg of p139 in enriched CFA, which contains 200 µg of heat-inactivated M. tuberculosis (seven mice), or infected with 4 × 106 CFU of living rMSp139, which corresponded to 200 µg of dry weight (six mice), or 4 × 106 CFU of living sham-transfected rMSHA (four mice). The total amount of p139 to which mice infected with rMSp139 are exposed during the 10 d following infection is thus estimated to be 4-fold that estimated for infection with 106 CFU of live rMSp139, i.e., 6.514 ± 0.055 ng/mouse, on average, with respect to 75 µg of peptide used in mice immunized with p139. The dry weight of mycobacteria was kept constant among groups in this experiment. EAE scores were then established according to the scale detailed in Materials and Methods for the following 67 d. Mice infected with rMSp139 actually develop a form of R-EAE similar to, although milder than, that induced by peptide immunization (Fig. 2A). The time of onset of the disease in the two groups overlapped (0.5 > p > 0.05). However, disease in the infected group reached a peak at day 22 and declined thereafter, whereas the average EAE score of the group of mice immunized with p139 increased until day 26. Beyond this time point, the differences between EAE scores of the two groups become statistically significant (0.05 > p > 0.005) and remained significant until disease remission was achieved in the mice immunized with p139, on average at day 33.4. Tail atony was observed in one of the mice infected with the control rMSHA strain on day 26 postinfection (and it lasted for 1 d) and a slight
Infection with live rMSp139 induces an R-EAE. A, SJL mice were immunized s.c. with 75 μg of p139 in enriched CFA (seven mice, △); six SJL mice were infected with 4 × 10^6 CFU of live rMSp139 (●) and four with 4 × 10^6 CFU of control rMS HA (○). EAE score was evaluated as described in Materials and Methods. Data report the average score for each group and SD. Student’s t test was performed at each time point between the scores observed for the group immunized with p139 and those observed in the group infected with live rMSp139. *p < 0.05; **p < 0.001. B, Three groups of SJL mice were infected with 10^6 (●, six mice), 4 × 10^6 (▲, six mice), and 4 × 10^7 (○, five mice) CFU of live rMSp139. EAE score was evaluated as described in Materials and Methods. Data report the average score and SD for each group. C, Incidence of EAE in SJL mice infected with the indicated CFUs of live rMSp139 (●) or immunized s.c. with 75 μg of p139 in enriched CFA/mouse (dashed bar). Groups comprise six mice (10^6 CFU), 12 mice (4 × 10^6 CFU), five mice (4 × 10^7 CFU), and 13 mice (p139), respectively. D, Histology and immunohistochemistry of 10-μm-thick transverse sections of spinal cord from representative SJL mice, immunized with p139 or infected with live rMSp139. Mice immunized with p139 (a–c) or infected with live rMSp139 (d–f) were sacrificed at day 24 or 22, respectively. CNS histology (a and d) and immunohistochemistry (b and e, anti-CD3; c and f, anti-Ricinus Communis Agglutinin I) were performed as described in Materials and Methods. Perivascular inflammatory lesions (a) and rich in CD3+ cells (b) and activated microglia (c) are evident in the white matter of mice immunized with p139. Only mildly demyelinating areas (d) without CD3-expressing lymphocytes (e) or activated microglia (f) are detected in mice infected with live rMSp139. Scale bar: 200 μm. E, CNS histology of SJL mice immunized with p139 or infected with live rMSp139 at relapse of disease. Mice immunized with p139 (a and b) or infected with live rMSp139 (c and d) were sacrificed at day 67, and CNS histology was performed as described in Materials and Methods. a and b, Periventricular inflammatory lesion with mononuclear cells infiltrating the adjacent white matter (capsula interna) (a) and meningeal and perivascular mononuclear infiltrates in the medulla oblongata (b) of representative mice immunized with p139; H&E staining of 10-μm coronal sections of the brain. Scale bar: 200 μm (a) and 100 μm (b). c and d, Representative longitudinal adjacent spinal cord sections from mice infected with live rMSp139. One area of demyelination is indicated by white arrows; H&E staining (c) or Wolcke for myelin staining (d). Scale bar: 150 μm.

FIGURE 2. Infection with live rMSp139 induces an R-EAE. A, SJL mice were immunized s.c. with 75 μg of p139 in enriched CFA (seven mice, △); six SJL mice were infected with 4 × 10^6 CFU of live rMSp139 (●) and four with 4 × 10^6 CFU of control rMS HA (○). EAE score was evaluated as described in Materials and Methods. Data report the average score for each group and SD. Student’s t test was performed at each time point between the scores observed for the group immunized with p139 and those observed in the group infected with live rMSp139. *p < 0.05; **p < 0.001. B, Three groups of SJL mice were infected with 10^6 (●, six mice), 4 × 10^6 (▲, six mice), and 4 × 10^7 (○, five mice) CFU of live rMSp139. EAE score was evaluated as described in Materials and Methods. Data report the average score and SD for each group. C, Incidence of EAE in SJL mice infected with the indicated CFUs of live rMSp139 (●) or immunized s.c. with 75 μg of p139 in enriched CFA/mouse (dashed bar). Groups comprise six mice (10^6 CFU), 12 mice (4 × 10^6 CFU), five mice (4 × 10^7 CFU), and 13 mice (p139), respectively. D, Histology and immunohistochemistry of 10-μm-thick transverse sections of spinal cord from representative SJL mice, immunized with p139 or infected with live rMSp139. Mice immunized with p139 (a–c) or infected with live rMSp139 (d–f) were sacrificed at day 24 or 22, respectively. CNS histology (a and d) and immunohistochemistry (b and e, anti-CD3; c and f, anti-Ricinus Communis Agglutinin I) were performed as described in Materials and Methods. Perivascular inflammatory lesions (a) and rich in CD3+ cells (b) and activated microglia (c) are evident in the white matter of mice immunized with p139. Only mildly demyelinating areas (d) without CD3-expressing lymphocytes (e) or activated microglia (f) are detected in mice infected with live rMSp139. Scale bar: 200 μm. E, CNS histology of SJL mice immunized with p139 or infected with live rMSp139 at relapse of disease. Mice immunized with p139 (a and b) or infected with live rMSp139 (c and d) were sacrificed at day 67, and CNS histology was performed as described in Materials and Methods. a and b, Periventricular inflammatory lesion with mononuclear cells infiltrating the adjacent white matter (capsula interna) (a) and meningeal and perivascular mononuclear infiltrates in the medulla oblongata (b) of representative mice immunized with p139; H&E staining of 10-μm coronal sections of the brain. Scale bar: 200 μm (a) and 100 μm (b). c and d, Representative longitudinal adjacent spinal cord sections from mice infected with live rMSp139. One area of demyelination is indicated by white arrows; H&E staining (c) or Wolcke for myelin staining (d). Scale bar: 150 μm.

Reduction of the hind footpad grasping was noticed on day 29–32 in one other mouse of this group; however, average disease score in the group infected with live rMSp139 was always significantly different from that of mice infected with control rMS HA strain during acute bouts of disease (e.g., day 26–32; 0.05 > p > 0.001).

In this experiment, all mice in both groups exposed to p139 reached an EAE score of at least 1. The average maximum score of disease was 2.29 ± 0.55 in mice receiving p139 in adjuvant versus 1.62 ± 0.35 in mice infected with rMSp139 (p = 0.027). Mice injected with p139 started relapsing at day 50 ± 3.4. rMSp139-infected mice also underwent a relapse of disease starting at day 57 ± 3.3 (p < 0.001). Average cumulative score over the 67 d of observation was 37.67 ± 10.38 in mice immunized with p139 and 18.23 ± 3.13 in mice infected with rMSp139 (p = 0.0034). Average duration of disease remission (calculated from the first day in which disease score was 1 point above the maximum value reached during the acute bout to the first day in which disease score had gained 1 point above the lowest value observed during remission) was 17.4 ± 4 d in mice immunized with p139 and 28.8 ± 5.2 d in mice infected with rMSp139 (p < 0.001), showing that disease remission was significantly longer in the disease induced by infection with rMS p139.

Induction of EAE by immunization with peptide depends on the dose of both peptide and adjuvant (15). To examine the effect of dose of infecting bacterium on the development of EAE, we conducted a second experiment with three groups of SJL mice, infected with 10^6 (6 mice) or 4 × 10^6 (six mice) or 4 × 10^7 (five mice) CFU of rMSp139. A fourth group of six mice was immunized with p139 in enriched CFA as positive control. Disease course was observed for 27 d. Average EAE scores for each group are reported in Fig. 2B. The incidence of disease development (mice developing an EAE score ≥ 1 ± total treated mice × 100), obtained pooling the observation of mice involved in the experiments reported in Fig. 2A and 2B, is reported in Fig. 2C. Two of six mice infected with 10^6 CFU of rMSp139 developed signs of disease starting on day 17 postinfection, with one mouse reaching a disease score of 1 and one other reaching 1.5, thereby indicating an incidence of the disease of 33% in this group. Average maximum score (1.30 ± 0.44 and 1.29 ± 0.40), incidence of the disease (five of six and four of five, respectively), and average day of onset of disease (18.2 ± 1.8 and 18 ± 2.2, respectively) did not differ between mice infected with 4 × 10^6 and 4 × 10^7 CFU of rMSp139. In the control group immunized with p139, the average maximum score was 1.8 ± 0.44 and five of six treated mice developed a score of disease ≥ 1 (data not shown). Thus, efficiency of induction of EAE by infection with rMSp139 depends on the dose of infecting bacteria used and reaches a maximum of severity and incidence at a dose of 4 × 10^6 CFU of rMSp139, in analogy to the observations showing that development of EAE requires an optimal dose of Ag and PPD and cannot be further improved by increasing either component of the mixture (15).
CNS examination was performed on mice immunized with p139 (three mice) or infected with rMS\textsuperscript{p139} (four mice) at the peak of disease (day 24–26 for immunized mice and day 21–22 for infected mice, respectively). All the mice immunized with p139 showed histological findings of EAE consisting of meningeal and perivascular mononuclear infiltrates in the white matter of the brain stem, rich in CD3\textsuperscript{+} cells and activated microglia (Fig. 2D–E). Infiltrates were not found in the CNS of mice infected with live rMS\textsuperscript{p139}, whose histological examination showed the presence of scattered areas of mild demyelination, mainly localized in the spinal cord (Fig. 2D–E). Similarly, distinct histological findings are observed also at relapse of disease (day 67; Fig. 2E), when we examined the mice infected with rMS\textsuperscript{p139} or immunized with p139 reported in Fig. 2A, that had reached the same score of disease. Thus, disease induced by infection with live rMS\textsuperscript{p139} displays a reduced inflammatory component and a dominant demyelination in comparison with disease induced by immunization with p139 in adjuvant. The histological findings in rMS\textsuperscript{p139}-infected mice intriguingly resemble histology of the CNS damage obtained by transfer of purified myelin-specific CD8\textsuperscript{+} cells (42) and are in accord with a less severe clinical course (43).

The number of rMS\textsuperscript{p139} CFUs in LN and spleen declines steadily after day 3 postinfection and rMS\textsuperscript{p139} was not detected in spleen or LN by day 20 postinfection (Fig. 1B). To further exclude that clinical symptoms were driven by the presence of rMS\textsuperscript{p139} in the CNS, two mice from the group infected with live rMS\textsuperscript{p139} were sacrificed at onset of their relapse (day 57). CNS and spleens from each mouse were then examined for presence of rMS\textsuperscript{p139}, as detailed in Materials and Methods. There was no evidence of bacterial growth or presence of ribosomal RNA of mycobacterial origin in either organ, indicating that development of clinical symptoms was independent of the presence of rMS\textsuperscript{p139} in the CNS and that rMS\textsuperscript{p139} had indeed been cleared by day 20 and was not present within the CNS.

Thus, infection with rMS\textsuperscript{p139} drives the development of clinical and histological demyelination independent of the presence of rMS\textsuperscript{p139} in the CNS. Acute and relapsing disease occurred after the clearance of the bacterium is achieved in peripheral lymphoid organs. However, the clinical course of the disease appears milder than what is observed after immunization with p139.

**Mice infected with rMS\textsuperscript{p139} modified their TCR repertoire specific for p139 but failed to expand the public BV10 T cells**

Challenge of SJL mice with p139 in adjuvant promotes a profound modification of the p139-specific TCR repertoire in SJL mice. The population of T cells responding to p139 shifts from a “pre-immune” repertoire (that comprises T cells carrying BV18-BJ1.2 [herewith with BV18] and BV19-BJ1.2 [herewith BV19] shared rearrangements) to a “postimmune” repertoire (that comprises BV10 cells, carrying a public BV10-BJ1.1 rearrangement, and BV4 cells, carrying a BV4-BJ1.6 semiprivate rearrangement). This new repertoire substitutes the previous one within LN and spleen. The reshuffling of the TCR repertoire follows specifically upon immunization with p139 (6). As described above, the amount of p139 available for immune recognition following infection with rMS\textsuperscript{p139} is 4 log lower than that used for peptide immunization. We therefore wondered whether this low amount of epitope is able to reshuffle the T cell response to p139 in a manner similar to peptide immunization.

We infected 12 SJL mice by s.c. injection of 10\textsuperscript{6} CFU of live rMS\textsuperscript{p139} that was equivalent on average to exposure to 1.628 ± 0.014 ng of p139. Nevertheless, infection with rMS\textsuperscript{p139} has a profound impact on the T cell repertoires specific for p139. The upper row of Fig. 3A shows that only 4 of 12 mice infected with live rMS\textsuperscript{p139} involved cells belonging to the p139-specific “pre-immune” repertoire (Fig. 3A, right column). This frequency is comparable to that seen in SJL mice after immunization with p139 in adjuvant as previously reported (6) and confirmed in this study by the data reported in the same Fig. 3A (one positive of four mice injected with p139 in adjuvant; Fig. 3A, left column). The spontaneous repertoire was depleted at the same time also from the spleens (data not shown). These data are consistent with the hypothesis that the p139-specific spontaneous repertoire was recruited and in part already depleted by rMS\textsuperscript{p139} to a similar extent as that seen following immunization with p139 in adjuvant (6).

At the same time point, the BV4 CD4\textsuperscript{+} cells that belong to the induced repertoire (28) are expanded in 4 of 12 infected mice, comparable to the frequency observed in mice immunized with p139 (Fig. 3A, lower row, right column, ●). However, only 1 of 12 mice infected with live rMS\textsuperscript{p139} had responding T cells carrying the public BV10 TCR rearrangement (Fig. 3A, lower row, middle column, ○), compared with >90% of mice that received the peptide in adjuvant (Fig. 3A, lower row, left column, ○, and Ref. 28). The BV10 cells were not detected in the spleen of the same mice, indicating that the failure in detecting these cells in the draining LNs was not due to their early egress from LN.

Because consistent induction of EAE in treated mice depends on infection with a minimum of 4 × 10\textsuperscript{6} CFU of rMS\textsuperscript{p139}, we also examined whether infection with this amount of live rMS\textsuperscript{p139} is needed to induce usage of T cells carrying the BV10 rearrangement. Thus, four SJL mice were infected s.c. with 4 × 10\textsuperscript{6} CFU of live rMS\textsuperscript{p139}, and immunoscope analysis was performed for the BV10 rearrangement. None of tested mice had responding T cells carrying the BV10 TCR rearrangement (Fig. 3A, lower row, right column). Therefore, inability of live rMS\textsuperscript{p139} to recruit BV10 T cells does not depend on the dose of the bacterium used for infection.

We examined the ability of DCs loaded in vivo with rMS\textsuperscript{p139} to stimulate expansion of BV10 T cells. As a source of DCs that had processed MPT64-p139 in vivo, female SJL mice were infected with rMS\textsuperscript{p139}, and 4 days later, DCs from the draining LNs were enumerated as described in Materials and Methods. This method results in a population enriched in DCs and able to stimulate p139-specific BV10 (and BV4) T cells without the need for addition of Ag in vitro (6, 29). As a source of BV10 and BV4 cells, three SJL mice were immunized with p139 emulsified in CFA. Ten days later, LNCs from individual mice were seeded at 5 × 10\textsuperscript{6} cells/well in 24-well plates in complete medium, in the absence of Ag (background), but in the presence of p139 (10 μg/ml) (positive control) or of 7.5 × 10\textsuperscript{4} DCs enriched from the LNs of SJL mice that had been infected with rMS\textsuperscript{p139} in vivo, without Ag added in vitro (test). The results of this experiment are shown in Fig. 3B. As expected, p139 added in vitro stimulated the expansion of the BV10 peak. However, expansion of the same peak was not observed when DCs loaded in vivo were used as the source of antigenic complexes, whereas T cells carrying the BV4 rearrangement were expanded by the same DCs (data not shown).

Two mechanisms can cause this selective failure of DC loaded of antigenic peptide postinfection with rMS\textsuperscript{p139} to recruit in vivo or expand in vitro BV10 cells. APCs infected by the bacterium may present the epitope recognized by BV10 cells so efficiently that activation induced cell death follows; or processing of MPT64-p139 fails to produce the MHC/peptide epitope that specifically activates the BV10 cells.

We therefore examined the ability of rMS\textsuperscript{p139} administered in vivo to expand BV10 and BV4 cells. If infection with rMS\textsuperscript{p139} results in overstimulation of BV10 cells, cells carrying this rearrangement should proliferate quickly, and expansion of the corresponding CDR3-β rearrangement will be detected by...
immunoscope after 36 h of stimulation. However, this expansion may be lost in samples collected after 72 h of coculture, because of activation-induced death of BV10 cells. Alternatively, if the epitope recognized by BV10 cells is not presented after processing of the MPT64-p139 secreted by rMSp139, expansion of the BV10 peak will not be detected at any time point.

Cells from the draining LNs of three SJL mice immunized with p139 in CFA (s.c.) were isolated as a source of BV10 and BV4 cells and cultured in the absence of Ag or in the presence of 10 μg/ml p139 (for 72 h, control) or 10^6 CFU of rMS p139 (for 36 or 72 h) (test). Cells were collected and immunoscope analysis for BV10 and BV4 rearrangements was performed. Results are shown in Fig. 3C. BV4 cells are expanded by rMS p139 added in vitro after 36 and (to a lesser extent) 72 h culture, indicating that the p139-derived epitope for which they are specific is available upon processing of MPT64-p139, in line with the ability of rMS p139 to prime in vivo BV4 cells. On the contrary, in all of the three mice, the BV10 TCR rearrangement is not expanded after stimulation with rMS p139 for 36 or 72 h, whereas it expands when LNCs are stimulated with p139.

Although we cannot formally exclude that cell death has already occurred by hour 36, these observations support the hypothesis that the epitope recognized by BV10 cells is distinct from what is recognized by BV4 cells and is not available on APCs following processing of MPT64-p139.

Shared CD8+ T cells specific for p139 expanded more efficiently postinfection with rMS p139 than following immunization with peptide in adjuvant

*M. smegmatis* primes efficiently CD8+ cells when used as vaccinia vector (24). CD8+ cells are among those spontaneously responding to p139 in naive SJL mice (6) and are deemed to be among the major players in human MS (44, 45). Therefore, we sought to determine whether a CD8+, p139-specific T cell repertoire is activated following immunization and whether infection with rMS p139 is also effective in expanding these cells.

SJL mice were immunized with 50 μg/mouse p139 in CFA or in enriched CFA. Eight days later, cells from draining LNCs were stimulated with p139 for 3 h and stained for Ag-driven IFN-γ secretion and CD8 expression as detailed in Materials and Methods. Results showed a robust detection of p139-specific CD8+ IFN-γ-secreting cells (Fig. 4A). The p139-specific stimulation index (i.e., the ratio between p139-stimulated versus background samples) ranged between 3 and 10. As a control of the specificity of the test, the stimulation index obtained by culturing the same LNCs in the presence of control peptide PLP178–191 ranged between 0.8 and 1.5 in three separate experiments (data not shown). The amount of *M. tuberculosis* in the adjuvant determined the number of IFN-γ-secreting CD8+ cells that increased from 200/10^6 IFN-γ-positive T cells in mice receiving the peptide in CFA to 400/10^6 in mice immunized in the presence of enriched CFA.

We examined the clonal composition of this CD8-mediated response by immunoscope. LNCs from immune mice were cultured in the presence or absence of p139. To account for the likely lower precursor frequency and proliferative ability of CD8+ cells compared with CD4+ cells, LNCs were cultured for 3 d, and CD8+ cells were then enriched in both samples by MACS sorting prior to performing a complete immunoscope analysis. Results from one representative mouse are shown in Fig. 4B, where seven TCR rearrangements contribute to the CD8-mediated response.

We then compared the spectra from six SJL mice and found that two rearrangements behaved as semiprivileged rearrangements, BV17-BJ1.6 of 139b length (herewith BV17 cells) being used by four of six mice and BV20-BJ2.3 of 104b length (herewith BV20 cells) by two of six mice. Representative spectra for the enrichment of BV17 and BV20 rearrangements in CD8+ cells are shown in Fig. 4C. Vice versa, the RSI of these two rearrangements in the total LNCs without enrichment for CD8+ cells usually falls below the significance threshold, and we detected only three significant expansions of one of these two rearrangements in 10 mice immunized (see Fig. 4E, left column).
Because Abs that specifically block the interaction between TCR and either K\(^\alpha\) or D\(^\alpha\) molecules are not commercially available, to confirm that BV17 and BV20 cells are indeed restricted by MHC class I-encoded molecules, we examined the ability of SJL MEFs (MEF-2) pulsed with p139 to support the expansion of these two rearrangements. MEF-2 (isolated and cultured as described in Materials and Methods) were seeded in a 24-well plate (5 x 10\(^4\) cell/well) in the absence or in the presence of 10 \(\mu\)g/ml p139 for 16-h. LNCs (5 x 10\(^6\)/ml) from three individual mice immunized with p139 were cultured in the absence or presence of p139. Three days later, CD8\(^+\) cells were selected from both samples, and a complete immunoscope analysis was performed, as described. Dashed and full squares indicate those private (dashed) or shared (full) BV-BJ rearrangements whose spectra showed Ag-driven expansion of one peak. C. Immunoscope spectra of BV17-BJ1.6 and BV20-BJ2.3 rearrangements obtained in total LNCs of mice immunized with p139 in CFA cultured in the absence of Ag (upper row) or in the presence of 10 \(\mu\)g/ml p139 (middle row) or from the same LNCs stimulated with p139 and enriched for CD8\(^+\) cells as described in Materials and Methods (lower row). Peaks corresponding to the shared rearrangements of 139b (BV17) and 104b (BV20) length are shaded. D. Cells obtained from the draining LNs of one representative SJL mouse immunized with p139 resuspended in enriched CFA were cultured in the absence or in the presence of p139 or in the presence of MEF-2 that had not or had been previously pulsed with 10 \(\mu\)g/ml p139, as described in Materials and Methods. Three days later, immunoscope analysis was performed for rearrangements of BV10-BJ1.1, BV17-BJ1.6, and BV20-BJ2.6. Peaks corresponding to the shared TCR rearrangements BV10 (97b), BV17 (139b), and BV20 (104b) are shaded. E. LNCs from SJL mice immunized s.c. with 50 \(\mu\)g of p139 in IFA containing 50 \(\mu\)g of M. tuberculosis (10 mice, left and right columns) or infected with 10\(^6\) of CFU (12 mice, middle column) were examined by immunoscope for expansion of the shared rearrangements BV17 (closed symbols) and BV20 (open symbols), following stimulation with p139 in vitro. In 9 of the 10 samples obtained from mice immunized with p139 in adjuvant, CD8\(^+\) cells were selected after stimulation with the Ag, before immunoscope analysis (right column). Results are reported as the RSI of each rearrangement in individual mice. \(\chi^2\) test was performed among the groups as indicated in the figure.

Because Abs that specifically block the interaction between TCR and either K\(^\alpha\) or D\(^\alpha\) molecules are not commercially available, to confirm that BV17 and BV20 cells are indeed restricted by MHC class I-encoded molecules, we examined the ability of SJL MEFs (MEF-2) pulsed with p139 to support the expansion of these two rearrangements. MEF-2 (isolated and cultured as described in Materials and Methods) were seeded in a 24-well plate (5 x 10\(^6\) cell/well) in the absence or in the presence of 10 \(\mu\)g/ml p139 for 16-h. LNCs (5 x 10\(^6\)/ml) from three individual mice immunized with p139 in enriched CFA were cultured in the absence of Ag or in the presence of p139 or with MEF-2 or with MEF-2 that had been pulsed for 16 h with p139 and thoroughly washed. Three days later, LNCs were harvested, and immunoscope analysis for BV10, BV17, and BV20 was performed. Results from one representative mouse are shown in Fig. 4D. As expected, the peak corresponding to the shared BV10 rearrangement is expanded in all three mice when p139 was added during in vitro culture. MEF-2 prepulsed with p139 do not support the expansion of BV10 cells (according to previously published data showing that they are CD4\(^+\) cells [28]), confirming that there is no presentation of p139 on class II-encoded molecules upon these experimental conditions. At the same time, the peaks corresponding to the shared BV17 and BV20 rearrangements are barely or not at all detected as expanded when p139 is present during in vitro culture of the total LNC population. However, when antigenic complexes are only provided by MEF-2 pulsed with p139, the expansion of these peaks is clearly detected, respectively, in two (BV17) and one (BV20) of three mice tested, indicating that T cells carrying these rearrangements are most likely restricted by MHC class I-encoded molecules.

Secretion of IFN-\(\gamma\) by CD8\(^+\) cells can play a role in EAE leading to the activation of microglia [46, 47]. To establish whether T cells carrying the BV17- and BV20-shared TCR...
rearrangements are able to secrete IFN-γ following Ag stimulation, LNCs from SJL mice immunized with p139 in CFA or enriched CFA were separated in populations enriched for cells secreting IL-10, IL-4, or IFN-γ, as described previously (6). As shown in Table I, BV17 cells are enriched in the IFN-γ-producing fraction following immunization in the presence of enriched CFA but not after immunization in the presence of regular CFA. Thus, BV17 cells appear amenable to modulation of their ability to secrete IFN-γ by the dose of M. tuberculosis, in contrast to what we previously observed for CD4+ p139-specific T cells carrying the shared BV10 and BV4 rearrangements (28).

Effector and effector/memory T cells are CD62Llow, CD45RA−, and CD45RO+, whereas naive and central/memory T cells are CD62Lhigh, CD45RA+, and CD45RO+. Both types of cells are present in lymphoid organs during immune responses (48). We enriched cells from draining LNs by magnetic immunoaffinity sorting for CD62Lhigh and CD62Llow, CD45RA+ and CD45RA−, and CD25+ and CD25− populations. Each population was then stimulated in vitro in the presence of p139 and B220+ cells enriched from the spleen of naive SJL mice and examined for presence of the shared TCR rearrangements associated with CD8+ p139-specific cells. Results in Table I show that both BV17 and BV20 cells display an effector/effector memory phenotype.

Finally, we tested the ability of infection with rMSp139 to expand the p139-specific CD8+ cells belonging to these repertoires (Fig. 4E). As described above, only three BV17 or BV20 rearrangements are detected as positive in total LNCs of 10 mice immunized with p139 and stimulated in vitro with this peptide (Fig. 4E, left column). When CD8+ cells were enriched, the number of expansions showing a RSI equal or >2 reached 10 (p = 0.0004, χ2 test), confirming that BV17 and BV20 rearrangements are associated with CD8+ p139-specific cells. Results in Table I show that both BV17 and BV20 cells display an effector/effector memory phenotype.

To confirm that infection with live rMSp139 recruits the same T cells that are enriched in the CD8+ population of LNCs immunized with p139, we sequenced the BV17 CDR3 regions from Ag-stimulated CD8+ LNCs of three mice immunized with p139 and from Ag-stimulated total LNCs of two mice infected with live rMSp139. A total of 120 plasmids were examined, and 75 contained in-frame products of rearrangement BV17-BJ1.6. Forty sequences display a 139b length and are shown in Table II. To identify a motif associated with TCRs specific for p139, we compared sequences of 139b length with those of other lengths (reported in Supplemental Table I). Residues P at position 1 following the CASS, G and S at position 4 and Y at the position that precedes the BJ-encoded NSPL appear selectively enriched in 139b sequences, whereas L at position 1, encoded by germline BV17 gene, appears to be selectively depleted. Although overall frequent, G at position 2 (that is possibly encoded by the DB region) is not enriched in 139b rearrangements. A candidate common motif for the p139-specific TCRs carrying the BV17-BJ1.6 of 139b length would therefore be CASS-1P-2 G-3×4G/S-5Y-NSPL. TCRs carrying this motif were found in two of three mice immunized with p139 and in both mice infected with rMSp139. 4G is preferred in mice immunized with p139, whereas 4S is preferred in mice infected with live rMSp139. In those rearrangements showing this motif, position 3 hosts preferentially positively charged residues (Q or N).

Despite a consistent effort (>40 plasmids were sequenced), we were unable to sequence any CDR3 of BV20-BJ2.3 from mice immunized with p139, and only five in-frame sequences were obtained from mice infected with live rMSp139 of 107b or 110b length (Supplemental Table I). Thus, at present, we cannot indicate a candidate shared motif for rearrangement BV20-BJ2.3 of 104b length.

p139-specific CD8+ cells appear in the CNS before disease onset, whereas appearance of CD4+ cells coincides with clinical bouts of EAE

We next asked whether CD4+ and CD8+, p139-specific T cells appear in the CNS at the same time during the various phases of R-EAE. EAE was induced in a group of 18 female SJL mice by injecting s.c. 75 μg of p139 resuspended in IFA supplemented with 200 μg/mouse of M. tuberculosis. Cells infiltrating the CNS were prepared from each mouse at various steps of disease, as described previously (6). Because we could identify more than one T cell for each repertoire used by a consistent fraction of SJL, we assumed that at least one T cell for each of the CD4 and CD8 repertoires would be used within a group of three mice. Therefore,

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**Table I. Cytokine secretion and expression of activation markers by shared CD8+ T cells specific for p139**

<table>
<thead>
<tr>
<th>Cytokine secretion</th>
<th>Expression of activation markers</th>
<th>ILN-Mice Immunized with p139 in CFA</th>
<th>Enriched CFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV17-BJ1.6 (150b)</td>
<td>N.T.</td>
<td>IFN-γ</td>
<td>N.T.</td>
</tr>
<tr>
<td>BV20-BJ2.3 (104b)</td>
<td>N.T.</td>
<td>CD25−, CD62Llow, CD45RA−, CD62Llow</td>
<td>N.T.</td>
</tr>
</tbody>
</table>

*p139-specific T cells secreting IFN-γ, IL-4, and IL-10 were enriched from LNs of SJL mice (immunized as indicated in the table) using MACS secretion kit, as described in Materials and Methods. Cells obtained from draining LNs were stimulated in the absence (background) or in the presence of 50 μg/ml p139, in a 6-well plate at a concentration of 5 × 10⁶ cells/ml. Three hours later, cells were harvested and submitted to the staining procedure described in Materials and Methods for each cytokine. Positively selected cells were collected and prepared for mRNA isolation for the TCR repertoire analysis. —, rearrangement not detected in any selected population.

Four SJL mice were immunized s.c. with p139 in enriched CFA, and 4 d later, cells from draining LNs were obtained. Cells were pooled and enriched by labeling LNCs with biotin-conjugated mAbs, followed by selection with streptavidin-conjugated MiniMACS beads. A total of 2.5 × 10⁶ positively or negatively selected cells were cocultured in vitro with 10⁶ B220+ cells selected from the spleens of naive SJL mice, in the presence or absence of 10 μg/ml p139 for 3 d. Immunofluorescence analysis for BV17 and BV20 T cells in all of the samples was performed as described in Materials and Methods.
CNS-infiltrating cells were obtained from three mice during the preclinical disease (day 11 after immunization); six at the onset of acute EAE; three after remission had been achieved; three upon relapse of disease; and finally, three during a late relapse or chronic disease. mRNA was prepared and cDNA was synthesized as described in Materials and Methods. The presence of the shared TCR-β rearrangements was examined by immunoscope in each CNS sample, and results are shown in Table III.

CD8+ T cells specific for p139 carrying the semiprivate TCRs described above (BV17 and BV20) were the first cells to appear in the CNS, albeit at a frequency lower than expected. In fact, they were detected in CNS samples obtained during preclinical and acute phases of EAE. However, we failed to detect them during the remission and relapse of EAE. One sample obtained during late/chronic disease was also positive for BV17 CD8+ cells.

We reported previously (6) that the public BV10 TCR (associated with IFN-γ-secreting, CD4+ T cells) was not detected in the CNS samples during preclinical disease. T cells carrying this rearrangement were consistently found in the CNS samples obtained during onset of acute disease (five of six mice), and the corresponding CDR3 could be sequenced out of CNS samples. In this study, we report that BV10 TCRs were not detected in the CNS during clinical remission of disease, although they could be found in the spleen of the same mice (data not shown). They were, however, detected again in the CNS during relapses (two of three in samples obtained during the first relapse and one of three in the samples obtained during the late/chronic disease). In contrast, T cells carrying the semiprivate BV4 rearrangement (CD4+, not secreting IFN-γ, IL-4, or IL-10) were never found in CNS.

Taken together, these observations suggest that p139-specific, CD4+ and CD8+ cells play distinct roles in R-EAE, with homing in the CNS of CD4+ BV10 cells consistently associated with the acute bout of symptoms.

Vaccination against BV10 CDR3 reduces usage of T cells carrying the BV10 rearrangement and diminishes the severity of the acute bout of EAE.

The observation that infection with rMSp139 does not activate the BV10 TCRs was not detected in the CNS during clinical remission of disease, although they could be found in the spleen of the same mice (data not shown). They were, however, detected again in the CNS during relapses (two of three in samples obtained during the first relapse and one of three in the samples obtained during the late/chronic disease). In contrast, T cells carrying the semiprivate BV4 rearrangement (CD4+, not secreting IFN-γ, IL-4, or IL-10) were never found in CNS.

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Vaccination against BV10 CDR3 reduces usage of T cells carrying the BV10 rearrangement and diminishes the severity of the acute bout of EAE.

The observation that infection with rMSp139 does not activate the BV10 T cells that home to the CNS in coincidence with the acute

Table II. Amino acid sequence of the CDR3 regions of T cells carrying rearrangements of BV17-BJ1.6 of 139b length in mice immunized with p139 in CFA or infected with rMSp139

<table>
<thead>
<tr>
<th>Progressive Mouse No.</th>
<th>Mice Challenged with</th>
<th>Source of T Cells</th>
<th>Sequences of 139b Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>p139 + CFA</td>
<td>p139-stimulated CD8+ cells*</td>
<td>CAS SPTNSYN5PLYFAA CAS SPTNSYN5PLYFAA CAS SPTNSYN5PLYFAA CAS SPTNSYN5PLYFAA CAS SLGTAYNSPLYFAA CAS SAGWYN5SLYFAA CAS SAGWYN5SLYFAA CAS SAGWYN5SLYFAA CAS SAGWYN5SLYFAA CAS SAGWYN5SLYFAA</td>
</tr>
<tr>
<td>2</td>
<td>p139 + CFA</td>
<td>p139-stimulated CD8+ cells*</td>
<td>CAS SPTNSYN5PLYFAA CAS SPTNSYN5PLYFAA CAS SPTNSYN5PLYFAA CAS SPTNSYN5PLYFAA CAS SAGWYN5SLYFAA CAS SAGWYN5SLYFAA CAS SAGWYN5SLYFAA CAS SAGWYN5SLYFAA CAS SAGWYN5SLYFAA CAS SAGWYN5SLYFAA</td>
</tr>
<tr>
<td>3</td>
<td>p139 + CFA</td>
<td>p139-stimulated CD8+ cells*</td>
<td>CAS SAGWYN5SLYFAA CAS SAGWYN5SLYFAA CAS SAGWYN5SLYFAA CAS SAGWYN5SLYFAA CAS SAGWYN5SLYFAA CAS SAGWYN5SLYFAA CAS SAGWYN5SLYFAA CAS SAGWYN5SLYFAA CAS SAGWYN5SLYFAA CAS SAGWYN5SLYFAA</td>
</tr>
<tr>
<td>4</td>
<td>rMSp139</td>
<td>p139-stimulated total LNCs*</td>
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*Cells obtained from draining LNs of three mice immunized with p139 resuspended in CFA were stimulated in vitro with p139 for 3 d. CD8+ cells were then selected by MACS immunoaffinity sorting, and BV17-BJ1.6 CDR3 sequences were obtained as described in Materials and Methods.

*Cells obtained from draining LNs of two mice infected s.c. with 10^6 CFU of live rMSp139 were stimulated in vitro with p139 for 3 d. BV17-BJ1.6 CDR3 sequences were obtained as described in Materials and Methods.
bout of EAE suggests that a failure to prime BV10 cells may contribute substantially to a milder course of the disease.

To test this hypothesis, we vaccinated SJL mice with a plasmid encoding for a CDR3 sequence corresponding to the public BV10 rearrangement, in tandem with IL-2 as adjuvant. We reported that DNA-based vaccination with a plasmid coding for the CDR3 region of an IgM μ-chain as a target Ag was able to induce an Ab response specific for the CDR3 of the μ-chain in two of three of vaccinated mice (49). We therefore inserted a sequence encoding for one CDR3-β belonging the BV10 public rearrangement (namely Y L C A S S P G T N T E V F F G) in the expression box of the published plasmid vector pRC110-NTS-IL-2 with IL-2 encoded in a separate expression box (34). The resulting plasmid vector was named pRC110-NTS-IL-2-BV10.

We first determined whether vaccination against the BV10 CDR3 region reduced the usage of the BV10 cells. Five mice were vaccinated by i.m. injection with the vaccine plasmid (pRC110-NTS-IL-2-BV10) and six with the sham plasmid (pRC110-NTS-IL-2, not encoding for the CDR3), as detailed in Materials and Methods. Both groups of mice were then immunized s.c. with p139 in CFA. Eight days later, immunoscope analysis was performed on cells isolated from draining LNs and stimulated in vitro with 10 μg/ml p139 for 3 d. Results (reported in Fig. 5A, open bars) showed that mice receiving the anti-CDR3 vaccine specific for the BV10 rearrangement use this rearrangement less efficiently than control mice (p = 0.046). This result was confirmed by cloning and sequencing the BV10 CDR3 region, looking for the CASS-SGS/PGS/PGT-NTEVFF sequences that we reported as associated with the public BV10 cells specific for p139 (28) (Fig. 5A, filled symbols, and Supplemental Table II). Confirming that the sham vaccination did not alter usage of the BV10 rearrangement, two mice treated with the control vector showed usage of these sequences (22 and 50% of 97b sequences, respectively). Six mice treated with the anti-CDR3 vaccine specific for the BV10 rearrangement were controlled for usage of CDR3. Only two mice use the public CDR3 (at a frequency of 16 and 50% of 97b sequences, respectively), whereas it was not possible to clone CDR3 of any public BV10 sequence from the other four vaccinated mice. Thus, sequencing of CDR3 confirms that usage of the public rearrangement is reduced in anti-BV10–vaccinated mice, with respect to control mice.

In two separate experiments, SJL mice were injected i.m. with control (pRC110-NTS-IL-2, six mice in total) or vaccine (pRC110-NTS-IL-2-BV10, five mice in total) plasmids, as described above. Ten days later, mice were immunized s.c. with 75 μg/mouse of p139 in enriched CFA. Mice were monitored daily for signs of EAE for the following 50 d, blind with respect to vaccine plasmid used. Results of one of the two experiments are shown in Fig. 5B, 5C. In this experiment, four mice were treated with the control and three mice with the BV10-containing plasmid, respectively.

Starting on day 6 after immunization with the peptide, mice from both groups developed a progressive increase of disease score, which may be due to the production of IL-2 encoded in the plasmid. All of the mice receiving the control plasmid pRC110-NTS-IL-2 had a rapid increase in clinical score (1 point of disease activity within 2 d) at day 19–20 (Fig. 5B). Disease score remained stable for 2–4 d and then declined (>0.5 point in 1 d). Thereafter, the score resumed climbing progressively during the following 25 d. Only two of the five mice that had been vaccinated with the vaccine plasmid pRC110-NTS-IL-2-BV10 (encoding for the CDR3 BV10 sequence in addition to IL-2) showed a rapid worsening of their clinical condition (Fig. 5C). Instead, disease score of the other mice of this group kept increasing gradually for the entire duration of the experiment. Comparing the results of the two experiments together for presence or absence of the rapid worsening of clinical score, the difference between the groups treated with control or BV10-containing plasmid is statistically significant (χ² test, p = 0.0009).

Taking these observations together with the data about the homing of BV10 cells into the CNS, results suggest a role for p139–specific BV10 cells in determining severity of the acute bouts of disease but not disease occurrence.

Discussion

In this study, we show that a transgenic M. smegmatis expressing a chimeric protein containing the self-epitope of p139 fused to MPT64 elicits an R-EAE that depends on the self-reactive response. Infection with rMSp139 modified the responding p139-specific T cell repertoire, depleting the p139-specific spontaneous repertoire, expanding efficiently p139-specific CD8+ cells, and activating CD4+ T cells carrying the BV4 semiprivileged rearrangement. In addition, BV10 public T cells were not activated following infection with rMSp139, because processing of the chimeric protein MPT64–p139 did not yield the epitope recognized by BV10 cells. Failure of rMSp139 to prime the BV10 cells is related to development of a milder disease, because SJL mice vaccinated with a plasmid encoding for a CDR3 sequence corresponding to the public BV10 rearrangement showed a reduced usage of the BV10 cells and a reduction of the acute bout of symptoms during EAE. Thus, peripheral infection with CNS-cross-reactive mycobacteria induces an R-EAE that continues after the infectious agent has been cleared. The composition of the self-reactive repertoire activated following infection determines the severity of the resulting disease.

We find that p139 expressed by an intracellular pathogen shows immunologic activity at a dose several orders of magnitude lower when compared with the same determinant administered as peptide. This observation supports the idea that intracellular bacteria are effective vaccinia vectors when a strong stimulation of the immune system is needed, such as, for example, in cancer vaccines (23). At the same time, it shows that transient encounters with infectious agents cross-reactive with self-produce profound and lasting effects on the immune system. Several lines of evidence indicate that an infectious environment can prevent the development of autoimmune diseases by modifying the self-specific T cell repertoire or by providing factors that influence Th polarization (50, 51). At the same time, acute rheumatic fever is one clearest example of a self-reactive response triggered by infectious agents (52). In this case, Abs and T cells specific for the M protein
Of Streptococcus cross-recognize heart proteins and cause a progressive damage of heart valves leading to deficient function (53).

Studies from Oldstone et al. (1) and Ohashi et al. (2) have shown that cross-reactivity between pathogens and self-proteins plays a critical role in the activation of self-reactive T cells in type I diabetes. Cross-mimicry between EBV-encoded nuclear Ag-1 and myelin Ag has also been demonstrated (54). Several infectious agents have been proposed to act as triggers or even causative agents of MS (13), and most of them directly infect the CNS where they can recruit pathogen-specific T cells and/or promote presentation of CNS Ags by infiltrating DCs.

Although this model of bacterial or viral Ags as trigger for autoimmune diseases is attractive (14), as yet no clear epidemiological evidence in favor of this hypothesis has been reported for most human T cell-mediated autoimmune diseases. Association of pathogens with self-reactive diseases is largely based on the detection of circulating Abs specific for the pathogen involved. As well, it is extremely difficult to study large cohorts of subjects for T cell responses. This poses a major limitation for investigating the role of infectious agents in triggering of autoimmune diseases, especially for agents that fail to induce consistent and stable levels of specific Abs, such as mycobacteria, mycoplasma, and Chlamydia. A role for this type of pathogen has been suggested by studies from Harkiolaki et al. (5), showing that peptides derived by microbial pathogens stimulate a T cells from a mouse transgenic for a TCR specific for DR2-MBP85–99. In the present report, we show that a self-reactive disease of the CNS can develop after a transient infection with a nonpathogenic Mycobacterium in the periphery, which results in appropriate stimuli leading to the development of the encephalitogenic phenotype in responding T cells (15).

Effective self-reactivity toward CNS proteins in fact also implies that T cells acquire the ability to cross the BBB. The relevance of this step in the pathogenesis of EAE is highlighted in naive SJL mice that display a large population of CD44+ p139-reactive T cells (11) and fail to develop EAE spontaneously, because these T cells lack the ability to cross the BBB (6). Likewise, it has been reported that healthy DR2+ subjects have circulating MBP-specific T cells, and yet show no sign of disease (55). We have reported that T cells reactive to human collagen are present in healthy donors and rheumatoid arthritis patients and belong to distinct repertoires (7).

Thus, self-reactive T cells detected in healthy subjects appear not to have pathogenic properties or may even have a protective role; induction of autoimmune diseases will require a profound modification of the self-reactive T cell repertoire and the acquisition of the appropriate homing properties that can be triggered by cross-reactive infectious agents. In addition, infection by a second pathogen may alter the BBB, analogous to pertussis toxin in EAE, thereby modulating disease development or severity.

The Ag-specific T cell repertoire selected in each individual response is modulated by several factors. The individual self-modifies the selection of T cells in the thymus (see Refs. 32 and 56). Immunological history shapes the T cell repertoire that will be involved in the response to a new pathogen through heterologous priming of T cells by cross-reactive pathogen(s) (57) or by exhaustion of immune repertoires (6, 58–60). We show in this study that differences in the molecular environment in which an epitope is contained resulted in a small variation of the determinants effectively presented. This small difference led to a distinct clonal composition of the activated T cell repertoire, despite an otherwise large cross-reactivity.

It is debatable whether the clonal composition of the T cell repertoire can affect the clinical outcome of EAE by influencing directly the balance of type 1, type 2, or type 17 responses. Some T cells appear to differentiate only into one phenotype, when activated by a fixed Ag (28, 61–63), indicating an intrinsic bias during the development of Th1 and Th2 phenotype (64, 65). At present there are little data regarding the role of TCR in determining the polarization of T cells toward Th17. Although polarization of the CD4 response specific for p139 in the SJL mouse is in part a function of T cell repertoire recruitment (28), we show in this study that shared CD8+ cells specific for the same peptide are amenable to modulation of their secretion profile by the adjuvant.
The role that CNS-specific CD8+ T cells play in MS and EAE is receiving increasing attention. (66, 67). CD8+ cells have been found in the plaques of MS patients. In these plaques, CD8+ cells can establish a contact with EBV-infected B cells. CD8+ T cells participate as regulators of the immune response during the course of EAE (69), but myelin-specific CD8+ cells also have a potential pathogenic role (70), especially in viral infections. CD8+ cells establish a contact with EBV-infected B cells (68). CD8+ cells are found in the plaques of MS patients (44, 45). In these plaques, IFN-γ-driven interactions (47, 75). Thus, CD8+ cells may act as triggers of acute EAE after immunization with p139. The induction of IFN-γ secretion in CD8+ cells may be particularly relevant (72) as IFN-γ secreted by CD8+ cells can activate microglia, thereby favoring activation of Th1 cells (46, 47). The failure to detect TCRs associated with CD8+ cells in the CNS at later time points, however, suggests that other mechanisms contribute to late stage damage of the myelin sheet, including B cell responses (76).

The lesions observed in the EAE triggered by infection with M. smegmatis show a damage of myelin and absence of CNS infiltration by blood-derived mononuclear cells. Activated CD8+ cells specific for CNS Ags induce demyelination more efficiently than CD4+ cells, in the absence of infiltration by blood-derived macrophages (42), and can kill target cells without bystander damage (47). Thus, it is possible that p139-specific CD8+ cells play a dominant role in the EAE induced by infection with live M. smegmatis that expands preferentially p139-specific cells of the CD8 lineage.

The encounter of pathogenic and nonpathogenic microbes profoundly modifies the immune system, with effects that can range from promotion to induction of autoimmunity. This report investigated the possibility that a self-sustaining autoimmune disease of the CNS arises and progresses following transient infection with a nonpathogenic bacterium despite the ability of the host to clear effectively the infectious agent.

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Disclosures
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References


