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23. Primary macrophages were lavaged from the peritoneal cavities of C57BL/6NCR mice 6 days after the intraperitoneal injection of 0.1 ml of sterile mineral oil, washed three times in Hanks' balanced salt solution (BioWhittaker Inc., Walkersville, MD), plated at $1 \times 10^5$ cells per well in complete medium [RPMI 1640 (Gibco BRL) supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), nonessential amino acids, 500 μM β-mercaptoethanol, and 10% fetal bovine serum (Hyclone Labs)], and allowed to adhere. Complete medium contained endotoxin (20 pg/ml) as determined by a chromogenic limulus amebocyte lysate assay (BioWhittaker). Nonadherent cells (~50% of the total) were washed off before infection. RAW 264.7 cells, L929 cells, and 293 cells (all from American Type Culture Collection) were plated at $2.5 \times 10^5$ cells per well in complete medium 24 hours before use. Some cultures were treated with pure recombinant mouse IFN-γ (25 U/ml) (for macrophages, RAW 264.7, and L929 cells) or human IFN-γ (for 293 cells) (Genentech) for 18 hours before and throughout infection. Virus was added at one plaque-forming unit (PFU) per cell and allowed to adsorb for 1 hour at 37°C before monolayers were washed three times with phosphate-buffered saline and repleted with 1 ml of complete medium. After 24 hours at 37°C, cells were scraped into culture supernatant, lysed by three cycles of freeze-thaw and sonication, and serially diluted 10-fold. Aliquots (0.5 ml) were adsorbed to BS-C-1 cell monolayers for 1 hour at 37°C and overlaid with Eagle's minimum essential medium with 10% fetal bovine serum and 0.9% (w/v) methylcellulose (Sigma Chemical). Viral plaques were counted on stained monolayers as described (*6*).
24. We thank J. Manning and M. A. Pospischil for amino acid analysis of culture media, L. Ignarro for SNAP, S. Shah and M. McCoss for NIO, G. Chaudhri for RAW 264.7 cells, M. Challberg for HSV-1, and Genentech, Inc., for IFN-γ. Supported by NIH grant CA43610 (to C.N.) and by the National Institute of Allergy and Infectious Diseases (to R.M.L.B., C.D., and G.K.).

23 March 1993; accepted 1 July 1993

# Helper T Cells Without CD4: Control of Leishmaniasis in CD4-Deficient Mice

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**Expression of either the CD4 or CD8 glycoproteins discriminates two functionally distinct lineages of T lymphocytes. A null mutation in the gene encoding CD4 impairs the development of the helper cell lineage that is normally defined by CD4 expression. Infection of CD4-null mice with *Leishmania* has revealed a population of functional helper T cells that develops despite the absence of CD4. These $CD8^-$ αβT cell receptor$^+$ T cells are major histocompatibility complex class II–restricted and produce interferon-γ when challenged with parasite antigens. These results indicate that T lymphocyte lineage commitment and peripheral function need not depend on the function of CD4.**

The critical role of T cells in the control of murine *Leishmania major* infection is underscored by the failure of the immune system of T cell–deficient nude or severe combined immunodeficient (SCID) mice to control fatal dissemination of the parasite (*1*). Sustained depletion of $CD4^+$ cells by monoclonal antibodies (mAbs) results in an inability to control disease (*2*), although transient depletion at the time of infection allows the outgrowth of protective type 1 helper T cells ($T_H1$ cells) in otherwise susceptible BALB/c mice (*3*). This requirement for $CD4^+$ cells may be related to the localization of the organism in a late endosomal compartment in macrophages into which major histocompatibility complex (MHC) class II molecules co-localize (*4*). Mice with a targeted disruption of the CD4 gene have impaired development of the CD4 lineage and decreased helper T cell activity (*5*, *6*). We examined the capacity of such mice to control an *L. major* infection in order to investigate the requirements for $CD4^+$ T cells in the control of this intramacrophage parasite.

Mice homozygous (−/−) and heterozygous (+/−) for the CD4 gene disruption on the (C57BL/6 × 129) $F_1$ ($H\text{-}2^b$) background were generated and screened (*6*). The parental and hybrid wild-type mice on this genetic background are resistant to infection with *L. major* and develop a small lesion at the site of inoculation that heals in 6 weeks. After receiving an inoculation of stationary-phase promastigotes in the hind footpads, both heterozygote and homozygote mice developed local swelling that peaked during the third to fourth week of infection and resolved thereafter (Fig. 1). Only 1 of 29 $CD4^{-/-}$ mice showed any delay in this pattern of resolution; that mouse had a footpad lesion of 5.1 mm after 9 weeks but was otherwise well. We cultured serial dilutions of footpad and splenic tissues to confirm that parasite replication had been controlled. Eleven infected $CD4^{-/-}$ mice had no recoverable promastigotes; only the animal with a persistent footpad lesion had recoverable parasites (from the footpad cultures only).

Resolution of *L. major* infection can be abrogated if the neutralizing antibody to interferon-γ (IFN-γ) is administered at the time of infection (*7*). When we administered intraperitoneally a single dose of neutralizing mAb to IFN-γ at the same time we inoculated mice with the parasite, healing was blocked in both control and $CD4^{-/-}$ mice. The lesions progressed (Fig. 1) and organisms were recovered from both footpads and spleen in all treated animals.

We assayed mice for delayed type hypersensitivity (DTH), a reaction attributed to $CD4^+$ $T_H1$ cells in normal mice (*8*), by injecting *L. major* antigens into one footpad 8 weeks after infection, by which time the local lesion had completely resolved. Saline was injected into the other footpad. At 48 hours, infected but not uninfected mice developed swelling typical for DTH only in the antigen-injected footpad (footpad thickness measured with a metric caliper was 2.18 ± 0.22 and 2.06 ± 0.18 mm for saline and 3.48 ± 0.24 and 3.28 ± 0.21 for 50 μg of *L. major* antigen in $CD4^{-/-}$ and

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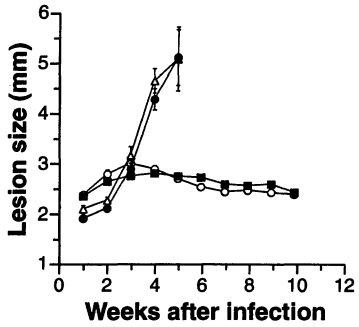


**Fig. 1.** Resolution of *L. major* infection in CD4-deficient mice. Groups of six mice with the designated +/− or −/− genotypes for CD4 were inoculated with 2 × $10^6$ stationary-phase promastigotes in the rear footpads, and the course of disease was monitored by measuring footpad thickness with a metric caliper. Two groups received a single dose of 1 mg of mAb XMG1.2 (anti–IFN-γ) on the day of infection. Results represent one of four comparable experiments. (○, $CD4^{+/-}$; ■, $CD4^{-/-}$; △, $CD4^{+/-}$ and anti–IFN-γ; ●, $CD4^{-/-}$ and anti–IFN-γ.)

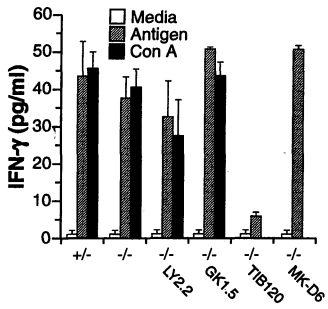


**Fig. 2.** Production of IFN-γ by lymphocytes from infected mice. Lymphocytes were collected and purified from the popliteal lymph nodes of mice that had been infected with *L. major* 5 weeks earlier. Cells ($10^6$ per milliliter) were incubated in triplicate in a final volume of 200 μl in either media, the mitogen Con A (5 μg/ml), or a lysate of *L. major* antigens (10 μg/ml) (first four grouped bars) or viable *L. major* promastigotes (5 × $10^5$ per milliliter) (two pairs of bars at the far right). Supernatants were collected after 48 hours and analyzed for IFN-γ by ELISA. +/−, $CD4^{+/-}$ heterozygote; −/−, $CD4^{-/-}$ homozygote; LY 2.2, infected mice treated twice weekly with mAb to CD8 throughout infection; GK1.5, infected mice treated twice weekly with mAb to CD4 throughout infection; TIB120, in vitro cultures supplemented with mAb M5/114.15.2 to I-$A^b$ (25 μg/ml); and MK-D6, in vitro cultures supplemented with mAb to I-$E^d$ (25 μg/ml). Fluorescence analysis confirmed that $CD4^+$ cells were absent in all $CD4^{-/-}$ animals and that anti-CD8 therapy reduced the numbers of $CD8^+$ cells from 11.8 ± 2.9 to 2.1 ± 0.4% (mean ± SEM) of total cells.

$CD4^{+/-}$ animals, respectively, mean ± SEM, $n = 5$).

The phenotypes of the lymphoid populations in the spleen of uninfected and infected $CD4^{+/-}$ and $CD4^{-/-}$ mice were examined by flow cytofluorometric analysis (Table 1). Compared with uninfected mice, infected $CD4^{+/-}$ mice had increased CD4/CD8 ratios and more B cells. Similar findings have been reported for other mouse strains (9). In the $CD4^{-/-}$ mice, splenic T cells were predominantly $CD8^+$, αβT cell receptor $(TCR)^+$, but there was also a significant population of $CD4^-CD8^-$ (double negative, DN), $TCR\alpha\beta^+$ cells. Infection of these mice significantly increased this DN population and increased the ratio of DN to $CD8^+$ cells. The numbers of γδ T cells, natural killer (NK) cells, and B cells in the spleen were comparable in both groups of mice.

Lymphocytes purified from recovered mice were incubated in vitro with parasite antigens or concanavalin A (Con A) and assayed for the production of IFN-γ after 48 hours (Fig. 2). Cells from $CD4^{-/-}$ and $CD4^{+/-}$ mice made comparable amounts of IFN-γ in an antigen-specific manner, whereas cells from uninfected animals generated no IFN-γ in response to parasite antigens. To address the potential role of $CD8^+$ T cells in controlling disease, we infected groups of $CD4^{-/-}$ animals with *L. major* and gave them weekly doses of mAbs to CD8 or CD4. In each case, the antibody did not affect the ability of the immune systems of these mice to control the infection. When cells from animals treated with the mAbs to CD8 or CD4 were stimulated in vitro with *L. major* antigens or Con A, the capacity of the cells to generate IFN-γ was not significantly different from that in untreated animals (Fig. 2). The production of IFN-γ was comparable if viable promastigotes were used instead of parasite lysates, and the production was abrogated in the presence of mAbs to MHC class II I-$A^b$ but not in the presence of mAbs to MHC class II of other specificities or an isotype control mAb (Fig. 2). Cells from uninfected mice generated no IFN-γ in vitro in response to parasite antigens.

The DN, $TCR\alpha\beta^+$ population was purified from infected $CD4^{-/-}$ mice with sequential magnetic bead selection and analyzed for IFN-γ mRNA transcripts by polymerase chain reaction (PCR) that was quantitated with a competitor construct (Fig. 3). Cytokine transcripts could be readily identified in the DN population and were comparable in amounts to the IFN-γ transcripts in the $CD4^+$ population purified from infected $CD4^{+/-}$ animals. The generation of IFN-γ, as measured by enzyme-linked immunosorbent assay (ELISA) after incubation of the cells with parasite antigens for 48 hours, was also comparable in these purified populations (*10*).

The abrogation of IFN-γ production by mAbs to MHC class II indicated that the DN cells from $CD4^{-/-}$ mice could recognize antigens in association with MHC class II, a characteristic of normal $CD4^+$ T cells. To better define the MHC restriction of the DN cells, we generated $CD4^{-/-}$ mice that were also deficient in expression of either $\beta_2$ microglobulin ($\beta_2$M) [and MHC class I (*11*)] or MHC class II (*12*). In $CD4^{-/-}$, $\beta_2M^{-/-}$ mice, the predominant population of T cells in the periphery was $CD8^-CD4^-$, $TCR\alpha\beta^+$ (Fig. 4A), similar to the population of DN cells in the $CD4^{-/-}$ mice. These mice readily controlled *Leishmania* infection (Fig. 4B) and contained antigen-specific MHC class II–restricted DN T cells (*10*). In contrast, mice lacking MHC class II were unable to control *Leishmania* (Fig. 4B), and this was not obviously exacerbated by the absence of CD4. This susceptibility

**Table 1.** Lymphocyte populations in mice infected with *L. major*. Spleen cells were collected from groups of five uninfected mice or five mice infected with *L. major* in the hind footpads 6 weeks previously, teased into single-cell suspensions, and incubated with mAbs to the designated phenotypes: CD4, GK1.5 conjugated to fluorescein isothiocyanate (FITC) (Becton Dickinson); CD8, YTS 169.4 conjugated to phycoerythrin (PE) (CalTag); TCRαβ, H57-597 conjugated to FITC (Pharmingen); TCRγδ, GL3 conjugated with PE (CalTag); and NK cells, PK136 (anti-NK 1.1) conjugated to PE. To identify DN, TCRαβ T cells, we incubated cells with markers for CD4, CD8, NK 1.1, B220, and TCRγδ (all conjugated to PE) and then incubated them with anti-TCRαβ conjugated to FITC (to allow gating on FITC-positive, PE-negative cells). Cells were analyzed on a Becton Dickinson FACScan with forward- and side-scatter analysis using LySys II software. Data represent mean percentage ± SEM of cells expressing the designated marker after scoring at least 5000 events. Results are from one of two independent experimental groups examined.

| Phenotype | Cells expressing the marker (%) | | | |
|---|---|---|---|---|
| | Uninfected mice | | Infected mice | |
| | $CD4^{-/-}$ | $CD4^{+/-}$ | $CD4^{-/-}$ | $CD4^{+/-}$ |
| CD4 | 0.1 ± 0.01 | 21.0 ± 0.6 | 0.1 ± 0.01 | 21.2 ± 1.3 |
| CD8 | 28.9 ± 1.9 | 15.6 ± 0.7 | 9.9 ± 1.9 | 5.6 ± 0.7 |
| TCRαβ | 38.4 ± 0.9 | 45.3 ± 2.4 | 33.4 ± 3.4 | 29.1 ± 2.5 |
| TCRγδ | 2.0 ± 0.3 | 2.1 ± 0.3 | 2.5 ± 0.2 | 2.6 ± 0.3 |
| DN, TCRαβ | 8.7 ± 0.2 | 2.0 ± 0.1 | 12.8 ± 0.9 | 2.7 ± 0.5 |
| B cell | 46.6 ± 0.8 | 44.3 ± 0.1 | 61.6 ± 2.4 | 55.2 ± 4.5 |
| NK | 0.7 ± 0.2 | 6.2 ± 1.0 | 2.1 ± 0.2 | 3.6 ± 0.8 |

to disease presumably reflects the absence of both MHC class II antigen presentation and parasite-responsive helper T cells. Serum collected after infection documented the ability of $CD4^{-/-}$ and ($CD4^{-/-}$ × $\beta_2M^{-/-}$) $F_1$ mice to generate a diverse antibody response to *L. major* that was similar in complexity to that of $CD4^{+/-}$ mice (Fig. 4C). Taken together, the results suggest that the DN cells in $CD4^{-/-}$ mice do not require MHC class I for their development and are likely to depend on MHC class II.

Although DN, TCR$\alpha\beta^+$ T cells are present in normal animals and constitute about 1% of the cells in the spleen, it is unlikely that this normal population is comparable to the DN cells mediating anti-*Leishmania* defense in $CD4^{-/-}$ mice. The DN T cells from normal mice are not subjected to typical positive and negative selection in the thymus (*13*). They may use a restricted $V_\beta$ repertoire with few junctional N regions (*14*) or consist of a subset of splenic T cells that serve as a major source of interleukin-4 (*15*). Preliminary analysis of $V_\beta$ expression by the $CD8^-$, TCR$\alpha\beta^+$ cells in the $CD4^{-/-}$ mice infected with *L. major* confirmed the presence of a diverse $V_\beta$ repertoire that did not differ significantly from the $CD4^+$, TCR$\alpha\beta^+$ repertoire in $CD4^{+/-}$ animals (*16*). Our finding that the $CD4^-$, TCR$\alpha\beta^+$ cells mediate all of the functions carried out by $CD4^+$ cells in $CD4^{+/-}$ littermates argues against expansion of a limited repertoire. The failure of otherwise normal mice to control infection after sustained depletion of $CD4^+$ cells (*2*) suggests that the normal DN T cells are unable to acquire the necessary phenotype to provide help for *Leishmania* infection.

The DN, TCR$\alpha\beta^+$ T cells in the $CD4^{-/-}$ mice recognized parasite antigens in association with MHC class II and did not require MHC class I for their development. In other experiments, the CD4 promoter was found to be active in the same population of cells in uninfected $CD4^{-/-}$ mice (*17*). Cumulatively, the data suggest that the CD4 lineage of T helper cells can develop without CD4 expression. At present, it is not clear what properties allow for development of these CD4-independent T cells. It is possible that these cells express relatively high-affinity TCRs or have more efficient coupling mechanisms for signal transduction, allowing for co-receptor–independent function. Alternatively, the cells that complete development may express other molecules that can substitute for CD4, such as the recently described CD4-related molecule, LAG-3, which is expressed on activated human T cells and binds to MHC class II (*18*).

The presence of $CD4^-$ T helper cells in the $CD4^{-/-}$ mice is consistent with recent results supporting a stochastic process of T cell lineage commitment (*19*). In this model, distinct signals through CD4 or CD8 are not required for progression of double positive thymocytes to single positive progeny. Instead, stochastic down-regulation of CD8 would be followed by positive selection of MHC class II–restricted cells. It has not been ruled out, however, that a molecule such as LAG-3 (*18*) could substitute for CD4 to deliver an instructive signal during thymic development.

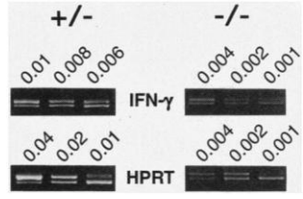


**Fig. 3.** Transcripts of IFN-γ in $CD4^{+/-}$ and $CD4^{-/-}$ lymphocytes. We collected lymphocytes from mice after 4 weeks of infection with *L. major* with anti-CD4–coated biotinylated magnetic beads in the $CD4^{+/-}$ mice (+/−, left column) and anti-TCR$\alpha\beta$ biotinylated magnetic beads in the $CD4^{-/-}$ mice (−/−, right column) after we repeatedly selected and removed cells that bound to anti-CD8– and anti-B220–coated beads. Transcripts for IFN-γ were analyzed and compared to transcripts for the constitutively expressed HPRT mRNA with a competitive reverse transcriptase-PCR assay (*20*). By this analysis, the ratio of IFN-γ to HPRT transcripts was about 0.4 (0.008/0.02) in $CD4^{+/-}$ cells from infected mice as compared with 1 (0.002/0.002) in $CD4^{-/-}$, TCR$\alpha\beta^+$ cells. Numbers represent the concentration of the competitor in nanograms per milliliter. Results represent one of three comparable experiments.

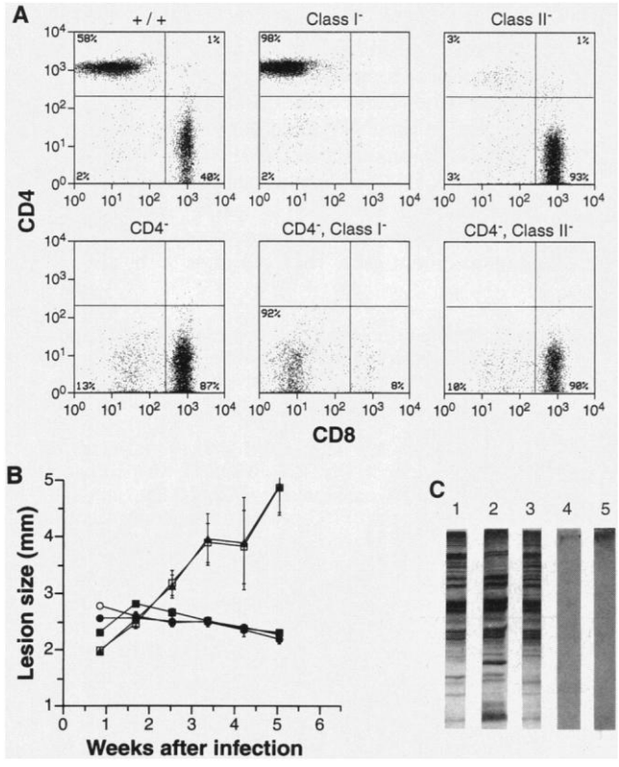


**Fig. 4.** Course of *L. major* infection in CD4-deficient mice. (**A**) Flow cytofluorometric analysis of CD4 and CD8 expression on T cells from lymph nodes of mice of the indicated genotypes. Cells were stained with anti-CD8 and anti-CD4 (53-6.7-FITC and GK1.5-PE, respectively, Becton Dickinson) and biotinylated anti-CD3ε (145-2C11, Pharmingen) followed by streptavidin-PE-Cy5 (CalTag Tricolor). We collected 10,000 events on a Becton Dickinson FACScan using LySys II software; plots show CD3-gated cells. (**B**) Groups of four $CD4^{-/-}$ (○), MHC class $II^{-/-}$ (□), MHC class $II^{+/-}$ (■), ($CD4^{-/-}$ × $\beta_2M^{-/-}$) $F_1$ (●), and ($CD4^{-/-}$ × MHC class $II^{-/-}$) $F_1$ (▲) mice were infected with *L. major*, and the course of infection was recorded with a metric caliper. (**C**) Complexity of parasite-specific antibody responses in CD4-deficient mice. Lysates of *L. major* were electrophoresed and transferred to nylon membranes. Protein immunoblots were incubated with sera from mice of the designated genotypes collected 5 weeks after infection. The immunoblot was washed, then further incubated with goat anti-mouse pan-IgG mAb conjugated to alkaline phosphatase (Promega), and developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. Lanes represent sera from the following mice: 1, $CD4^{+/-}$; 2, $CD4^{-/-}$; 3, ($CD4^{-/-}$ × $\beta_2M^{-/-}$) $F_1$; 4, MHC class $II^{-/-}$; and 5, ($CD4^{-/-}$ × MHC class $II^{-/-}$) $F_1$.

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20. The mRNA was purified from positively selected cells with RNAzol (Biotecx) and reverse-transcribed with random hexamer primers, deoxynucleotide triphosphates (Boehringer Mannheim), and murine Moloney leukemia virus reverse transcriptase (BRL) which served as the PCR template. Amplification was carried out in the presence of primers specific for IFN-γ (5′-CGGCACAGTCATTGAAAGCCTAG; 3′-GGCGCTGGACCTGTGGGTTGTTG) and the constitutively expressed gene hypoxanthine-guanine phosphoriboxyl transferase (HPRT) (5′-GTTGGATACAGGCCAGACTTTGTTG; 3′-GAGGGTAGGCTGGCCTATAGGCT). We used a competitor construct containing the IFN-γ and HPRT amplification products into which an additional sequence was inserted to generate a larger transcript (S. L. Reiner, S. Zheng, D. B. Corry, R. M. Locksley, *J. Immunol. Methods*, in press).
21. Supported by grant AI30663 from the National Institute of Allergy and Infectious Diseases of NIH, the Burroughs Wellcome Fund, the Markey Foundation, and the Howard Hughes Medical Institute. We thank D. Mathis and R. Jaenisch for supplying transgenic mice, K. Boyd for technical assistance, and A. Weiss for advice in review of the manuscript.

13 April 1993; accepted 12 July 1993

# MHC-Restricted Depletion of Human Myelin Basic Protein–Reactive T Cells by T Cell Vaccination

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**Activated autoreactive T cells are potentially pathogenic and regulated by clonotypic networks. Experimental autoimmune diseases can be treated by inoculation with autoreactive T cells (T cell vaccination). In the present study, patients with multiple sclerosis were inoculated with irradiated myelin basic protein (MBP)–reactive T cells. T cell responses to the inoculates were induced to deplete circulating MBP-reactive T cells in the recipients. Regulatory T cell lines isolated from the recipients inhibited T cells used for vaccination. The cytotoxicity of the $CD8^+$ T cell lines was restricted by major histocompatibility antigens. Thus, clonotypic interactions regulating autoreactive T cells in humans can be induced by T cell vaccination.**

Circulating autoreactive T cells can be isolated from the blood of normal individuals (*1*). The activation of autoreactive T cells may lead to their pathogenicity in the induction of autoimmune diseases (*2*, *3*). For example, in multiple sclerosis (MS), a chronic inflammatory disease of the central nervous system (CNS) characterized by infiltration of T cells and macrophages, an increased frequency of activated as opposed to resting MBP-reactive T cells is found in patients with active disease (*4*). These autoreactive T cells are probably clonally expanded and accumulate in the MS lesions (*5*). The mechanism by which MBP-autoreactive T cells are regulated in the disease is unknown.

Experimental autoimmune encephalomyelitis (EAE), a paralytic disease of the CNS resembling MS, is an animal model to investigate the regulation of autoreactive T cells (*6*). Small numbers of activated, but not resting, MBP-reactive T cells can rapidly induce the disease on adoptive transfer into naïve animals (*7*). These pathogenic T cells can also be isolated from unprimed rats to mediate lethal EAE (*8*). The MBP-reactive T cells are part of the normal T cell repertoire and may naturally be regulated by clonotypic networks (*9*, *10*). The regulation is postulated to involve recognition of T cell receptor (TCR) $V_\beta$ chains in the context of major histocompatibility complex (MHC) molecules (*10*, *11*). This concept has led to the paradigm of T cell vaccination, in which attenuated MBP-autoreactive T cells are used to up-regulate clonotypic regulatory networks to prevent and treat EAE (*12*).

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We investigated the nature of T cell–specific clonotypic responses to autologous MBP-autoreactive T cells in vivo and whether the responses are effective in depleting circulating MBP-reactive T cells. These experiments were carried out in patients with MS because regulation of MBP-reactive T cells has potential therapeutic consequences in the disease.

Six patients with clinically definite MS participated in this phase 1 trial. Three patients were diagnosed as having relapsing-remitting MS, and three other patients had primary and chronic progressive MS. The MBP-reactive T cell lines were first generated from subjects and then cloned by limiting dilution (*13*). Clinical data of the patients and the fine specificity of the T cell clones used as inoculates are shown in Table 1. In agreement with previous studies (*14*, *15*), this panel of T cell clones reacted to at least four different peptides, with a predominant reactivity pattern to peptide 84–102 and peptide 143–168. Thus, selection of T cell clones for inoculation was based on their reactivity to a predominant peptide, which varied between subjects. For example, three T cell clones reactive to peptide 84–102 were selected for subject GE, since all the clones isolated from this subject responded exclusively to this peptide. Thus, in each case at least two T cell clones reactive either to a predominant MBP peptide or to different epitopes (for example, the clones from BC) were pooled as a vaccine. T cell clones were activated with MBP-pulsed antigen-presenting cells (APCs) 4 days before injection (*16*). A total of three inoculations were given to each recipient by injection of a pool of two or three irradiated T cell clones ($10^7$ to $1.5 \times 10^7$ cells per clone) subcutaneously (*16*).

T cell responses to the inoculates were examined and compared with nonspecific phytohemagglutinin (PHA)-induced autologous T cell blasts prepared concurrently (*17*). All six subjects developed a substantial proliferative response to the inoculates after the second inoculation. The responses were accompanied by a marginal reactivity to PHA-induced T cell blasts (Fig. 1). We examined the frequency of MBP-reactive T cells before and after each inoculation of irradiated MBP-reactive T cell clones (*17*). This revealed a progressive decline of circulating MBP-reactive T cells, most notably after the second inoculation. The decrease in the frequency of MBP-reactive T cells correlated reciprocally with the responses to the inoculates (Fig. 1). The frequency fell below the detectable limit of our assay in five of the six recipients by the end of the trial. MBP-reactive T cells in subject HM could still be detected after the third inoculation, but at a frequency ($1.1 \times 10^{-7}$) that was only 20% of the preinocu-