γδ T Cells Enhance the Expression of Experimental Autoimmune Encephalomyelitis by Promoting Antigen Presentation and IL-12 Production

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γδ T Cells Enhance the Expression of Experimental Autoimmune Encephalomyelitis by Promoting Antigen Presentation and IL-12 Production

Artur Odyniec,* Marian Szczepanik,† Marcin P. Mycko,* Mariusz Stasiolek,* Cedric S. Raine,‡ and Krzysztof W. Selmaj*‡

Using an adoptive transfer model of experimental autoimmune encephalomyelitis (EAE) induced by myelin basic protein (MBP)-reactive lymph node cells (LNC), we have shown that depletion of γδ T cells from LNC resulted in diminished severity of EAE in recipient mice, both clinically and histopathologically. The reduced potency of γδ T cell-depleted LNC to induce EAE correlated with decreased cell proliferation in response to MBP. The γδ T cell effect upon the threshold of MBP-induced LNC proliferation and EAE transfer was restored by reconstitution of γδ T cells derived from either MBP-immunized or naive mice, indicating that this effect was not Ag specific. The enhancing effect of γδ T cells on MBP-induced proliferation and EAE transfer required direct cell-to-cell contact with LNC. The γδ T cell effect upon the LNC response to MBP did not involve a change in expression of the costimulatory molecules CD28, CD40L, and CTLA-4 on TCRαβ+ cells, and CD40, CD80, and CD86 on CD19+ and CD11b+ cells. However, depletion of γδ T cells resulted in significant reduction in IL-12 production by LNC. That γδ T cells enhanced the MBP response and severity of adoptive EAE by stimulating IL-12 production was supported by experiments showing that reconstitution of the γδ T cell population restored IL-12 production, and that γδ T cell depletion-induced effects were reversed by the addition of IL-12. These results suggest a role for γδ T cells in the early effector phase of the immune response in EAE. The Journal of Immunology, 2004, 173: 682–694.

E xperimental autoimmune encephalomyelitis (EAE)3 is an animal model of multiple sclerosis (MS) (1). It is an inflammatory demyelinating disease of the CNS characterized by oligodendocyte damage, myelin loss, and axonal damage (2). EAE can be actively induced by immunization with the myelin Ags myelin basic protein (MBP) (3), proteolipid protein (4), or myelin/oligodendrocyte glycoprotein (5), and the disease can be adoptively transferred to a healthy recipient with syngeneic T cells sensitized to myelin Ags (6). It is known that CD4+ T cells play an instrumental role in the adoptive transfer of EAE (7, 8). However, CD8+ T cells (9), B cells (10), NK cells (11), and γδ T cells (12, 13) are also known to contribute to the disease process.

γδ T cells constitute a minor population of T cells (14), with a TCR structure different from that of αβ T lymphocytes (15). Another important difference between αβ and γδ populations is the manner in which they recognize Ag. Although recognition of some Ag by γδ T cells is MHC restricted (16–19), others (like phosphoantigens and peptides derived from heat shock proteins (hsp)) do not require the presence of professional APCs and MHC (18–21). In addition, MHC molecules are not always involved in the differentiation and maturation of γδ T cell populations, in contrast to αβ lymphocytes (18–23). Accordingly, γδ T cells can develop extrinsically, but the mechanism of their selection is not fully understood (18, 19, 22, 23).

γδ T cells are known to eliminate infected or neo-transformed cells, due to their strong cytotoxic activity (24). However, aside from this effector function, γδ T cells seem to be important regulatory elements of the immune system (25). It has been shown that γδ T lymphocytes are capable of enhancing inflammatory responses in autoimmune diseases, e.g., systemic lupus erythematosus, rheumatoid arthritis (25, 26), graft-vs-host disease (27), and delayed-type hypersensitivity (28, 29). In addition, γδ T cells are involved in the mechanism of immune tolerance induced by oral (30) or high-dose i.v. (31) Ag administration. Their immunoregulatory function has been suggested to be dependent on interactions with T lymphocytes responding to Ag (32), and with the determination of the Th1/Th2 balance (19, 32, 33), by influencing the secretion of numerous cytokines, e.g., IFN-γ, IL-2, IL-4, IL-5, IL-10, and GM-CSF (18, 19, 34). γδ T cells share many features with another regulatory cell subset: NK cells. Both populations are characterized by high expression of inhibitory and stimulatory MHC class I receptors and can be activated by pattern-type ligands (35).

A role for γδ T cells in the pathogenesis of autoimmune demyelination has long been suspected. The accumulation of T cells bearing γδ TCR has been found in MS (36, 37) and EAE (12, 38). It has been shown that γδ T cells are cytotoxic for oligodendrocytes, the glial cells responsible for the synthesis and maintenance of myelin (39). Depletion of the γδ T population with anti-γδ T cell Abs, in mice sensitized for EAE by adoptive transfer of encephalitogenic T cells, reduced the severity of clinical EAE and of

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3 Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; MBP, myelin basic protein; hsp, heat shock protein; m, murine; LNC, lymph node cell; L, lumbar vertebra; SI, stimulation index; w/h, wild type.

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inflammation in the CNS (12). This effect was associated with decreased production of the proinflammatory cytokines IL-1, IL-6, TNF-α, and IFN-γ (40), and the chemokines MIP-1α and MCP-1 (41). The influence of γδ T cells on EAE has been confirmed with mice deficient for TCR δ-chain (δ−/−) (13). EAE induced with myelin/oligodendrocyte glycoprotein in δ−/− mice was less severe, and production of proinflammatory cytokines was significantly reduced. Although contradictory results have been published (42), collectively, these data suggest a role for γδ T cells in the regulation of EAE, but the significance and mechanisms responsible for this regulation remain unknown.

In the present work, we have shown, for the first time, that γδ T cells were required for the successful adoptive transfer of EAE and for the MBP-induced proliferative response in vitro. These data support the concept that γδ T cells supplement the encephalitogenic function of αβ T cells by the stimulation of IL-12 production by APCs.

Materials and Methods

Animals

Six- to 10-week-old SJL/J, B10.PL (The Jackson Laboratory, Bar Harbor, ME), and B10.PL TCRα−/− female mice (a gift of Dr. C. Janeway Jr., Department of Immunobiology, Howard Hughes Medical Institute, Yale University, New Haven, CT) were used for experiments. Animals were housed and maintained in an accredited facility, the Animal Care Department of Medical University of Lodz. A total of 453 mice was used for these experiments.

Reagents

MBP, Mycobacterium tuberculosis, RPMI 1640, penicillin/streptomycin mixture, glutamate solution, sodium pyruvate solution, HEPEs, nonessential and essential amino acid solutions, 2-ME, PBS, BSA, α-phenylenediamine dihydrochloride, and 30% H2O2 were purchased from Sigma-Aldrich (St. Louis, MO); CFA, from Difco (Detroit, MI); rabbit complement lyophilisate, from Biotech (Dreieich, Germany); FCS, from Invitrogen Life Technologies (Carlsbad, CA); recombinant human IL-2, from CytoTech (Copenhagen, Denmark); recombinant murine (m)IL-12, from CytoTech (London, U.K.) and from Genzyme (Cambridge, MA); and peroxidase-conjugated streptavidin, from Vector Laboratories (Burlingame, CA).

mAbs and reagents for FACS analysis

The following mAbs against mouse cell markers were used: supernatants containing mAb anti-pan-TCRγδ (UC7 13D5) from Dr. J. Bluemstein (University of Chicago, Chicago, IL); supernatants containing mAb anti-TCRβ (H5-597) from Dr. R. Kubo (University of Colorado, Denver, CO); R-PE-conjugated mAb anti-pan-TCRγδ (GL3), FITC-conjugated mAb anti-CD3, R-PE-conjugated mAb anti-CD3, PerCP-Cy5.5-conjugated mAb anti-CD25, FITC-conjugated streptavidin, R-PE-conjugated rat mAb anti-CD4, R-PE-conjugated rat mAb anti-CD8, R-PE-conjugated rat mAb anti-CD86, R-PE-conjugated hamster mAb anti-CTLA-4, R-PE-conjugated anti-CD25 mAb, R-PE-conjugated anti-CD69 mAb, R-PE-conjugated anti-CD44 mAb (all from BD Pharmingen, San Diego, CA); R-PE-conjugated hamster mAb anti-CD40L (from Biosource International, Camarillo, CA); R-PE-conjugated rat mAb anti-CD40, biotinylated anti-pan-TCRγδ (GL3), FITC-conjugated hamster mAb

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Depletion of γδ T cells before adoptive transfer reduces the severity of EAE. The EAE was induced by passive transfer of LNC, from mice previously immunized with MBP. Recipients (n = 18) received 8 × 10^7 LNC incubated with rabbit complement (○), or γδ-depleted LNC (●). γδ T cell depletion was performed in vitro before LNC transfer by incubation with anti-pan-TCRγδ UC7 13D5 and then with rabbit complement. Expression of disease was determined according to the clinical scale described in Materials and Methods. The figure demonstrates the daily mean clinical index (+SD) in both examined groups. Signs of EAE were significantly reduced in mice that received γδ-depleted cells (p < 0.05).

Table 1. Clinical and histopathologic correlation

<table>
<thead>
<tr>
<th>Animal</th>
<th>Expt. Group</th>
<th>Day of Onset/Day of Sampling</th>
<th>Maximum Clinical Score/Score at Time of Sampling</th>
<th>Histopathology*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>γδ-depleted LNC</td>
<td>13/20</td>
<td>1.0/0</td>
<td>Inflam. 0 0 0</td>
</tr>
<tr>
<td>2</td>
<td>γδ-depleted LNC</td>
<td>13/20</td>
<td>2.0/1.5</td>
<td>Demyel. 1 1 2</td>
</tr>
<tr>
<td>3</td>
<td>Total LNC</td>
<td>12/20</td>
<td>3.0/1.5</td>
<td>W.D. 3 4 2</td>
</tr>
<tr>
<td>4</td>
<td>Total LNC</td>
<td>12/20</td>
<td>4.0/3.0</td>
<td></td>
</tr>
</tbody>
</table>

* Inflammation (Inflam.), demyelination (Demyel.), and wallerian demyelination (W.D.) were scored on a 5-point scale based on intensity and extent. In all animals, note how lower clinical score correlated with decreased histopathologic findings, and in no. 4 (total LNC), how the higher score correlated with severe nerve fiber degeneration, whereas in no. 3 (total LNC), the lower score correlated with a more demyelinating disease.
anti-TCRαβ, FITC-conjugated rat mAb anti-CD19, FITC-conjugated rat mAb anti-CD11b, R-PE-conjugated hamster mAb anti-CD28, and R-PE-conjugated rat mAb anti-CD80 (all from Caltag, San Francisco, CA). The following control isotypes were used: FITC-conjugated hamster IgG1, R-PE-conjugated hamster IgG1, and R-PE-conjugated rat IgG2a (all from BD Pharmingen); and FITC-conjugated rat IgG2a and R-PE-conjugated rat IgG2b (both from Caltag).

**Immunization with MBP**

Mice were immunized with whole MBP protein as previously described (12). Briefly, 800 μg of protein (per mouse) was suspended in 100 μl of distilled water and mixed with 100 μl of CFA. This mixture (200 μl per mouse) was injected into two sites over the flanks. After 10–15 days postimmunization, animals were sampled, and brachial, axillary, and inguinal lymph nodes were removed for lymph node cell (LNC) culture.

**Depletion of γδ T cells**

Lymph nodes isolated from MBP-immunized mice were disrupted using a cell strainer (Falcon, New Haven, CT), and LNC were obtained by a series of washings in PBS at 4°C. The depletion procedure was performed by incubation with 1 ml of supernatant (containing not less than 10 μg/ml anti-pan-TCR-γδ) per 1 × 10⁷ cells for 45 min at 4°C. LNC were then washed twice with cold PBS, and 1 × 10⁷ cells/ml were incubated with rabbit complement (42). As a control, LNC were incubated with rabbit complement alone. The complement was prepared from a suspension of lophylisate in 10 ml of PBS, 15 min before the assay at room temperature. LNC were incubated with rabbit complement for 60 min at 37°C, and the cells were then washed twice and resuspended at the concentration needed in T cell culture medium. An alternative method for γδ depletion was negative selection using the MACS sorting system. LNC (1 × 10⁷) were incubated with biotinylated anti-pan-TCR-γδ mAb GL3 (Caltag) for 10 min at 4°C. After double washing with PBS containing 5% BSA, LNC were then incubated with anti-biotin microbeads for 15 min at 4°C. Next, LNC were washed three times and suspended in 500 μl of PBS/5% BSA. The beads coated with cells were applied onto a MS column (Miltenyi Biotec, Auburn, CA) that was placed in the magnetic field of a MiniMACS separator (Miltenyi Biotec). The negative fraction (γδ⁺) was collected. For both methods used, the depletion of γδ T cell was confirmed by flow cytometry using a FACSscan (BD Biosciences, San Jose, CA).

**Positive selection of γδ T cells by magnetic bead cell separation**

γδ T cells were isolated from the whole LNC population by a magnetic bead cell selection technique. LNC from previously immunized animals or naïve SJL/J mice were isolated as described above. For positive selection of γδ T cells, 1 × 10⁷ LNC were incubated with 1 μg of biotinylated anti-pan-TCR-γδ Ab for 10 min at 4°C. After double washing with PBS containing 5% BSA, LNC were then incubated with anti-biotin microbeads for 15 min at 4°C. Following this, LNC were washed three times and suspended in 500 μl of PBS/5% BSA. The beads coated with cells were applied onto an MS column (Miltenyi Biotec) that was placed in the magnetic field of a MiniMACS separator (Miltenyi Biotec). The column was then removed from the separator, and the positive (γδ⁺) cells were collected. The purity of the isolated cells was tested by FACS analysis after staining with FITC-conjugated streptavidin.

**Induction of EAE**

For induction of EAE, LNC derived from MBP-immunized SJL/J mice were cultured in T cell culture medium (RPMI 1640 medium containing 10% FCS, 100 μg/ml penicillin/streptomycin mixture, 2 mM glutamate, 0.1 mM sodium pyruvate, 20 mM HEPES, 1% nonessential and 0.5% essential amino acids, and 0.05 mM 2-ME). Cells were plated at a density of 4 × 10⁶/ml in 24-well plates at 37°C in 5% CO₂ and stimulated with MBP (50 μg/ml) (12). γδ T cells were depleted before or after 3 days of culture, depending on the experimental conditions, as described above. In some sets of experiments, depleted γδ T cells were replaced by the same number of γδ T cells isolated (as described above) from lymph nodes of naïve or previously MBP-sensitized, syngeneic mice. γδ T cells and γδ deplemented LNC were mixed and cultured together in a two-chamber system using Transwell 0.2-μm anapore membrane (Nunc, Naperville, IL) to separate both populations from each other. Reconstitution of γδ T cells was made before incubation with MBP or shortly before transfer of LNC to recipient mice. In some experiments of EAE, rmIL-12 (PeproTech, London, U.K.) at 1 μg per 10⁶ cells was added to γδ-depleted LNC. In all of the experiments, transfer populations were counted and injected into the tail vein of syngeneic, naïve recipients at the same number, 8 × 10⁷ cells per mouse (12). Mice were observed for 20 days post-passive transfer. Clinical score was estimated in both groups according to a previously published clinical scale from 0 to 5: 1, limp tail; 2, hindlimb weakness; 3, plegia of both hindlimbs; 4, tri- or tetraplegia; and 5, moribund or death (12, 13).

**Histopathology**

Four representative animals (two receiving γδ-depleted LNC and two receiving nondepleted total LNC) were sampled 20 days after adoptive transfer per perfusion with 20 ml of cold-buffered 2.5% glutaraldehyde. Each sampled pair comprised one animal displaying a mean clinical score for the group and one, a maximum score (see Fig. 1 and Table I). Brain and spinal cord were dissected from each animal, and 1-mm slices were taken from...
the cerebral hemispheres, cerebellum/brainstem, and the spinal cord at cervical vertebra 7, thoracic vertebra 2, lumbar vertebra (L)2, L5, L6, and sacral vertebra 1. In addition, optic nerves and spinal nerve roots were taken. For light microscopy, the tissue was postfixed in 1% osmic acid, washed, and dehydrated through a graded series of ethanol, cleared in propylene oxide, and embedded in Epon 812 (Electron Microscopy Sciences, Fort Washington, PA). One-micrometer sections were cut from the epoxy-embedded tissue and stained with 1% toluidine blue. Inflammation, demyelination, and wallerian degeneration were scored on a 5-point scale (12).

T cell proliferation assay

LNC, from MBP-immunized animals, or LNC depleted of γδ T cells, were incubated for 72 h with MBP (10–200 μg/ml). The proliferative response of positively selected γδ T cells to MBP was also investigated using macrophages or irradiated (5000 rad) LNC as APC. In each experiment, 2 × 10^5 cells per well were cultured in 96-well plates with a U-shaped bottom at 37°C. After 48 h, 100 μl of supernatants was collected and stored at −80°C for cytokine analysis. Proliferation was measured by the addition of 1 μCi of [methyl-^3]H]thymidine (Amersham, Arlington Heights, VA) to each well. After 24 h, cells were harvested on glass fiber filters (Skatron, Sterling, VA), using a semiautomated multichannel harvester (Skatron). Counts were determined by scintillation in a beta counter (LKB Pharmacia, Uppsala, Sweden). Results of the proliferation assay were estimated as stimulation indices (SI) according to the following formula: SI = count in investigated sample/count in negative-control sample.

Reconstitution of γδ T cell population and IL-12 addition

For reconstitution of the γδ population, 1 × 10^7 γδ-depleted LNC were mixed directly with 5 × 10^6 γδ-positive cells, or to avoid direct contact, these two populations were separated from each other by a Transwell 0.2-μm anopore membrane (Nunc). The proliferation assay with MBP was then performed, as described above. The effect of IL-12 on the MBP response of γδ-depleted LNC was also investigated by proliferation assay after the addition of rmIL-12 (PeproTech) in concentrations from 10 pg/ml to 1 ng/ml.

FACS analysis

Cells were stained with FITC- or PE-conjugated Abs. Aliquots of 1 × 10^5 cells were stained with 1 μg of two or three different Abs, one to determine the type of cell (anti-CD3, anti-TCRγδ, anti-TCRαβ, anti-CD19, anti-CD11b), and the second and third to detect specificity for a particular costimulatory or activation molecule on the cell surface (see mAbs and reagents for FACS analysis). Cells were washed twice after staining, and 100 μl of PBS plus 5% BSA was added to each sample. Analysis of stained cells was performed by a FACSscan flow cytometer and CellQuest software (BD Biosciences).

ELISA

 Supernatants from LNC cultures were collected after 48-h stimulation with PHA (5 μg/ml) or MBP (100 μg/ml), IL-12, IL-10, and IFN-γ production was tested by ELISA, as described previously (30). Briefly, ELISA plates (Corning, Corning, NY) were coated with the purified monoclonal rat Abs anti-IL-12p40, anti-IL-10, or anti-IFN-γ (all from BD Pharmingen). Next, the supernatant sample solutions (50 μl) and standard solutions of the particular cytokines under examination were added (rmIL-12 from Genzyme, rmIL-10 from BD Pharmingen, and rmIFN-γ from PeproTech). The secondary Ab was biotinylated rat mAb (BD Pharmingen; or Endogen, Woburn, MA). After washing, peroxidase-conjugated streptavidin (Vector Laboratories) was added. The assay was developed with peroxidase substrates: o-phenylenediamine (Sigma-Aldrich) and 30% H₂O₂ (Sigma-Aldrich), and read after the addition of stop solution (3 M H₂SO₄) at λ of 492 nm, using a plate reader (Bio-Rad, Hercules, CA). Cytokine concentrations were determined from standard curves established with recombinant standards.

Isolation and culture of peritoneal thioglycolate-induced macrophages

Peritoneal exudate cells were induced by i.p. injection of 2 ml of thioglycolate medium (Difco). Three days later, mice received i.p. PBS, beads coated with UC7 or H57 mAb, or 2 × 10^6 isolated T γδ or T αβ cells from the spleens of naive mice. The following day, peritoneal exudate cells were isolated by washout with 5 ml of Dulbecco’s PBS containing heparin at 5 U/ml. Thioglycolate-induced peritoneal cells contained >95% macrophages (FeR⁺ and esterase⁺ cells) and were not further purified. Macrophages were suspended in RPMI 1640 supplemented with FCS (1 × 10^5/ ml) and cultured in triplet in 24-well flat-bottom plates (Falcon) in a 5% CO₂ incubator. After 24 h, the medium was harvested and frozen at −80°C until use for IL-12 measurement.

FIGURE 3. Fluorescence flow cytometry analysis of γδ T cell depletion. A, Whole population of LNC was isolated from SJL mice. γδ T cell depletion was performed by negative selection with using MACS sorting system (8) or by incubation with UC7 13DA mAb in complement-mediated manner (C) as described in Materials and Methods. Cells were staining with FITC-conjugated anti-CD3 mAb and R-PE-conjugated anti-pan-TCRγδ GL3 mAb. The results are presented as fluorescence histograms, with the CD3 staining shown on x-axis and TCRγδ staining on the y-axis using a log/log scale.
Statistical analysis

Student’s t and Cochran-Cox tests were used to assess the significance of differences between groups. A value of $p < 0.05$ was considered significant.

Results

Mice given $\gamma\delta$ T cell-depleted LNCs develop less severe EAE

The number of $\gamma\delta$ T cells in the LNC population after depletion was at least 3-fold lower, compared with nondepleted LNC populations, and the mean number (three experiments, six mice in each) was 0.6 ± 0.4%. Transfer of $\gamma\delta$-depleted MBP-reactive cells resulted in the development of less severe EAE compared with transfer with the whole population of MBP-reactive LNC (Fig. 1). Time of disease onset was similar in both groups, but incidence of EAE in the control group was 100%, whereas in mice that received $\gamma\delta$ T cell-depleted LNC, incidence was only 60%. Mean clinical score was significantly lower in the $\gamma\delta$ T cell-depleted group when compared with EAE induced with nondepleted MBP-reactive LNC. In addition, histopathologic findings taken from four representative animals (Table I) indicated that, in mice receiving $\gamma\delta$ T cell-depleted LNC, although day of onset was similar to that of the nondepleted group, inflammation and demyelination were either not detectable (Fig. 2, A and B; Table I) or minimal, in which diffuse nerve fiber damage was noticeable (C and D; Table I). In

FIGURE 4. $A$, $\gamma\delta$ T cell depletion reduces LNC proliferation in response to MBP. LNC were derived from MBP-immunized mice and depleted of $\gamma\delta$ cells as described in Materials and Methods. Nondepleted LNCs and LNC incubated with rabbit complement were used as controls (●; ■). Each point represents the mean SI (± SD). Depletion of $\gamma\delta$ T cells (▲) significantly reduced MBP-induced LNC proliferation ($p < 0.05$). $B$, Similarly, when $\gamma\delta$ T cells were depleted from LNC population with MACS (□) (see Materials and Methods), the MBP-induced proliferation was significantly reduced, compared to control LNC (○). $C$, $\gamma\delta$ T cells do not respond to MBP. $\gamma\delta$ T cells were isolated from LNC of MBP-immunized SJL/J mice by positive selection technique (using mAb anti-pan-TCR$\gamma\delta$ and MACS sorting system). $\gamma\delta$ T cells were mixed with APC (5000-rad irradiated LN cells or monocytes isolated from LNC suspension by an adherence assay) at a ratio of 1:1 and stimulated with MBP for 72 h. Bars demonstrate mean SI ± SD. $D$, Presence of $\alpha\beta$ T cells is critical for MBP-induced LNC proliferation. LNC were isolated from MBP-immunized B10.PL w/t and TCR$\alpha^{-/-}$ B10.PL mice. Proliferation after MBP stimulation was observed only in LNC isolated from w/t animals (■), whereas LNC from TCR$\alpha^{-/-}$ mice did not proliferate after MBP stimulation (□).
contrast, in animals receiving total (nondepleted) LNC, the neuropathologic outcome was much more severe. Lesions invariably were extensive and reached the dimensions of discrete plaques, which in less-affected animals were inflammatory and highly demyelinating, being characterized by prominent preservation of axons and less conspicuous nerve fiber (wallerian) degeneration (Fig. 2, E and F; Table I). In contrast, in the more severely affected example, a broad zone of white matter damage completely encircled the spinal cord, widespread macrophage activity and wallerian degeneration were in evidence, and beginning glissois was present (Fig. 2, G and H; Table I). These results indicated that γδ T cells play an important enhancing role in the transfer and induction of EAE.

Depletion of γδ T cells diminishes MBP-specific LNC proliferation in vitro

To investigate the mechanism involved in the enhancing effect of γδ T cells in the adoptive transfer of EAE, we assessed LNC proliferation in response to MBP before cell transfer. The mean number of γδ T cells in the LNC population after complement-mediated depletion (0.43 ± 0.22%) or after negative selection by MACS sorting system (0.12 ± 0.04%) was significantly reduced compared with nondepleted LNC (1.62 ± 0.52%). The result of a representative depletion experiment is presented in Fig. 3.

The mean SI for MBP was significantly lower in γδ T cell-deficient population of LNC obtained by complement-mediated depletion (1.2 ± 0.1), compared with control LNC (2.0 ± 0.15 for nondepleted and 2.05 ± 0.2 for complement added; Fig. 4A; p < 0.05). The reduced proliferation of γδ-depleted LNC was also confirmed by experiments with negative selection of γδ T cells, by sorting with magnetic beads and MACS system (Fig. 4B).

To determine whether the effect of γδ T cell depletion on MBP-induced proliferation was not associated with their innate ability to proliferate in response to MBP, we assessed proliferative responses of isolated γδ T cells stimulated with MBP. γδ T cells did not proliferate in response to MBP (Fig. 4C). To exclude the possibility that the anti-TCR mAb used for γδ cell isolation might block recognition of MBP, we performed experiments with TCRα chain knockout mice. The population of T cells in these animals is represented by only γδ T cells. LNC isolated from MBP-immunized TCRα−/− mice did not respond to MBP, in contrast to LNC obtained from wild-type (w/t) animals (Fig. 4C). Additionally, in experiments with blocking of αβ TCR, with H57-597 mAb, in LNC culture, we were able to suppress MBP-induced LNC proliferation, despite full accessibility of TCR on γδ T cells (1.06 ± 0.2 vs 2.04 ± 0.32 in culture without H57-597 mAb). The lack of MBP-induced proliferation of γδ T cells is also supported by the lack of an increase in their number in LNC proliferation after MBP stimulation (Fig. 5A). We have also seen no increase in expression of activation markers like CD25, CD69, and CD44 (Fig. 5B). All these data strongly suggest that γδ T cells facilitated MBP-induced proliferation of LNC rather than proliferating themselves.

We found also that the diminished MBP-induced proliferative response of γδ-depleted LNC correlated with a decreased encephalitogenicity of these cells. We were not able to induce EAE by transfer of LNC depleted of γδ T cells before stimulation with

**FIGURE 5.** A, Fluorescence flow cytometry analysis of γδ T cell frequency in LNC population before culture and after 72-h culture with no-Ag (unstimulated) or with MBP. Cells were staining with FITC-conjugated anti-CD3 mAb and R-PE-conjugated anti-pan-TCRγδ GL3. The results are presented as fluorescence histograms, with the CD3 staining shown on the x-axis and TCRγδ staining shown on the y-axis using a log/log scale. B, Fluorescence flow cytometry analysis of CD25, CD69, and CD44 expression on γδ T cell from unstimulated LNC, LNC stimulated with MBP, or Con A. C, Fluorescence flow cytometry analysis of CD25, CD69, and CD44 expression on γδ T cell in control LNC or LNC incubated with anti-TCRγδ mAb and microbeads. Cells were staining with PerCP-Cy5.5-conjugated anti-CD3 mAb, FITC-conjugated anti-pan-TCRγδ GL3 and PE-conjugated anti-CD25 mAb, or anti-CD69 mAb or anti-CD44 mAb. The results are presented as fluorescence histograms, with the CD3/TCRγδ population gating, and CD25, CD69, or CD44 staining on the y-axis using a log/log scale.
To determine whether the direct interaction between LNC and MBP (data not shown). These findings confirm previous observations that encephalitogenic potential of immune cells is connected with their ability to respond to myelin Ags (43, 44).

Reconstitution of γδ T cells restores MBP-induced proliferation of LNC in vitro and their ability to transfer EAE

To reconfirm the facilitating function of γδ T cells in the immune response to MBP, we attempted to reverse the defective response of LNC depleted of γδ T cells by reconstitution of γδ T cells. γδ T cells were positively selected from both MBP-immunized and naive mice. The proportion of TCRγδ+ cells after positive selection comprised 55 ± 11% of total LNC. γδ T cell-depleted LNC were mixed with positively selected γδ T cells in proportions similar to the physiologic range of γδ T cells (<5% total T cells). γδ T cell reconstitution restored the ability of γδ-depleted LNC to respond to MBP stimulation. Both γδ T cells isolated from naive and MBP-immunized mice were able to restore the effect of depletion. The SI of LNC with reconstituted γδ T cells and of LNC without γδ T cell depletion were comparable and significantly higher than that of γδ-depleted LNC (Fig. 6, A and B). These results reconfirmed a role for γδ T in the immune response to MBP. Because there was no difference in the restoration of responsiveness to MBP with γδ T cells derived from naive or MBP-immunized mice, it appeared that the γδ T cell-facilitating effect was not Ag specific. To address the potential role of γδ T cell activation by anti-TCR mAb during their purification, we assessed (by flow cytometry) the expression of activation markers CD25, CD69, and CD44 on γδ T cells, before and after incubation of LNC with GL3 mAb, and found no difference (Fig. 5C).

Similarly to the results of MBP-induced proliferation assays, we observed that γδ T cells were able to restore the encephalitogenicity of γδ-depleted LNC. The expression of EAE induced by adoptive transfer of LNC with reconstituted naive γδ T cell population was considerably higher than in mice that received γδ-depleted LNC. (Fig. 7).

γδ T cell-facilitating effect on MBP immune response and EAE transfer requires cell-to-cell contact

To determine the role of soluble factors and/or the requirement of direct interaction between γδ T cells and LNC during the MBP response, we performed experiments with an anopore membrane to separate γδ T cells from the γδ-depleted population of LNC. The percentage of γδ T cells derived from MBP-immunized mice and γδ-depleted LNCs were similar to those which demonstrated successful restoration of MBP response after γδ T cell reconstitution (see above). Separation of γδ T cells from LNCs by an anopore membrane abrogated the facilitating effect of γδ cells on the immune response to MBP (Fig. 6C). These data suggested that direct cell-to-cell contact between γδ T cells and LNC was required for the MBP response.

The separation of γδ T cells and γδ-depleted LNC by anopore membrane during culture with MBP also abrogated totally the ability of these LNC to induce adoptive transfer EAE (data not shown). Even when γδ T cells were separated from LNC during 3 h before adoptive MBP transfer, the ability of EAE was comparable with severity of EAE induced by transfer with γδ-depleted LNC (Fig. 7).

All these observations confirm a crucial role of direct contact between γδ T cells and other LNC in regulation of MBP-specific response and induction of EAE.

γδ T cells do not affect costimulation signals

To determine whether the γδ T cell-facilitating effect on MBP responsiveness depended on costimulation, we investigated

FIGURE 6. Reconstitution of γδ T cell population restores the effect of MBP-induced proliferation of LNC. γδ T cells obtained by positive selection as described in Materials and Methods from immunized (○) and naive (□) mice were mixed with LNC depleted of γδ T cells. The percentage of γδ T cells in the reconstituted population corresponded to the proportion before depletion. Each point represents mean SI (+ SD). A, Nondepleted population of LNC (◇) and LNC incubated with rabbit complement (■) and γδ T cell-depleted LNC (▲) were used as controls. B, Reconstitution with both γδ T cells from either immunized or naive mice restored MBP-induced LNC proliferation (p < 0.05). C, MBP proliferation was restored only when direct contact between γδ T cells and LNC depleted of γδ T cells was present (○), and not when γδ T cells and LNC depleted of γδ T cell were separated by an anopore membrane (x) (p < 0.05).
whether depletion of γδ T cells affected expression of costimulatory molecules on T and B cells and APC (CD11b
+ cells) within LNC populations. The proportions of TCRαβ
+, CD19
−, and CD11b
+ cells in LNC populations were comparable before and after depletion of γδ T cells (data not shown). γδ T cell depletion also did not influence the frequency of CD4
+ and CD8
+ T cells (data not shown). The level of CD28 expression on αβ T cells was slightly reduced after γδ depletion, but the difference was not statistically significant (p > 0.05; Fig. 8A). The expression of other costimulatory molecules (CD40L, CTLA-4) on T cells remained unchanged after γδ T cell depletion. Similarly, γδ T cell depletion did not change CD40, CD80, and CD86 expression on B cells (Fig. 8B). The expression of CD80 on APC was slightly higher in non-depleted than in γδ-depleted LNC, but this difference was not significant (p > 0.05; Fig. 8C). The expression of CD40 and CD86 on CD11b
+ cells was comparable in control and γδ T cell-depleted LNC. Thus, γδ T cell depletion did not influence the proportion of other immune cells and did not affect the expression of costimulatory molecules on the surface of T, B, and CD19
+ cells.

γδ T cell-facilitating effect in MBP immune response depends on IL-12 production

The level of IL-12 production was significantly reduced after depletion of γδ T cells in LNC stimulated with MBP and PHA. The average concentrations of IL-12 in supernatant from γδ T cell-depleted LNC were 2.5-fold lower than those from control LNC (Fig. 9A). These results implicated a role for IL-12 in the mechanism by which γδ T cells facilitated the effect on MBP- and PHA-induced responses of LNC. The reconstitution of γδ T cells led to restoration of IL-12 production by LNC (Fig. 9B), a property that correlated with restoration of MBP-induced proliferation of LNC. Accordingly, the addition of IL-12 to γδ T cell-depleted LNC restored LNC proliferation in response to MBP (Fig. 9C). The levels of other cytokines (IL-10, IFN-γ), were not changed in cultures of LNC after depletion of γδ T cells (data not shown).

To confirm further a role for γδ T cell in inducing IL-12, we investigated whether γδ T cells affected IL-12 production by APC in mice in vivo. Intraperitoneal injection of γδ T cells into mice with previously induced peritoneal exudate resulted in a significant (p < 0.05) increase in IL-12 production by peritoneal macrophages, whereas injection of αβ T cells did not (Fig. 10).

Moreover, the reduced EAE transfer with γδ-depleted LNC was compensated for by addition of IL-12. Stimulation of γδ-depleted LNC with IL-12 for 3 h restored their ability to evoke EAE transfer (Fig. 11).

All of these data demonstrate a significant role for IL-12 in the regulatory mechanism of γδ T cells in the induction of adoptive transfer EAE.

Discussion

In this report, we have assessed the immunoregulatory role of γδ T cells in a model of EAE induced by the transfer of CD4
+ MBP-reactive T cells to naive mice. We have shown that the presence of γδ T cells enhanced the potency of encephalitogenic cells to transfer EAE and led to more destructive CNS lesions, and facilitated recognition of encephalitogenic Ag, MBP. The mechanism underlying the EAE-enhancing effect appears to depend upon the induction of IL-12 production by γδ T cells.
Our results on the contribution of γδ T cells to the transfer of EAE are in agreement with previous findings showing less severe EAE in animals deficient for γδ T cells (12, 13). However, in these studies, the demonstration that the deficiency in host γδ T cells was associated with less severe EAE, and correspondingly minor lesion activity, was interpreted as representative of a role for γδ T cells in late effector mechanisms of the disease. Accordingly, γδ T cells were shown to have strong cytotoxic effects upon oligodendrocytes in vitro (38) and stimulated the release of proinflammatory cytokines and chemokines in EAE (13, 40, 41). In keeping with this cytotoxicity was the observation that animals receiving nondepleted LNC had higher clinical scores and more prominent lesion activity, the milder scores being associated with less destructive inflammatory lesions in which primary demyelination was the major feature, and the most severe of which were accompanied by widespread CNS lesions that were highly destructive, leading to total loss of nerve fibers. In addition to a proposed effector function, γδ T cells have also been shown to possess important immunoregulatory activities (25–32). For example, in contact hypersensitivity, the presence of γδ T cells was responsible for

FIGURE 8. Expression of costimulatory molecules by LNC is not affected by γδ T cell depletion. γδ T cell-depleted LNC (○) and control LNC treated with rabbit complement (■) were cultured with or without MBP for 48 h. Cells were then collected and stained with mAbs: anti-pan-TCRαβ conjugated with FITC and anti-CD28, anti-CD152 (CTLA-4), or anti-CD154 (CD40L) (all conjugated with R-PE) (A); anti-CD19 conjugated with FITC and anti-CD80 (B7-1), anti-CD86 (B7-2), and anti-CD40 (all conjugated with R-PE) (B); anti-CD11b mAb conjugated with FITC and anti-CD80 mAb, anti-CD80 (B7-1) mAb, and anti-CD40 (all conjugated with R-PE) (C). Immunostaining was assessed by flow cytometry. Bars represent mean ± SD of the proportion of positive cells for each given marker.
transfer of hypersensitivity to naive animals (29). This function of γδ T cells was explained by their possessing antisuppressor activity (29). The present results have demonstrated that depletion of γδ T cells reduced effective transfer of EAE and indicated that γδ T cells operated in conjunction with encephalitogenic T cells in the early effector phase of the disease. Because endogenous γδ T cells in adoptively transferred animals did not compensate for those that were removed from the donor population, it appears that very early and intimate contact between γδ and γδ T cell population in the transferred LNC is required to maintain their encephalitogenicity.

To determine the mechanism of γδ T cell interactions with encephalitogenic T cells, we have shown in this study that γδ T cells facilitate MBP-induced proliferation in vitro before cell transfer. Because γδ T cells alone did not respond to MBP, this suggested that they might influence MBP immune responsiveness by providing additional signals directed either at MBP-reactive CD4+ T cells or at APC present in the population of encephalitogenic cells used for the transfer of EAE.

T cells of the γδ lineage are highly potent producers of proinflammatory cytokines (18, 19, 34), which propagate and enhance the Th1-type response, a response instrumental in the induction of EAE. However, the γδ T cell-assisting effect on the MBP-specific immune response and EAE transfer required direct cell-to-cell contact and was prevented when γδ T cells were physically separated from encephalitogenic T cells. Thus, direct interaction between surface molecules of γδ T cells and other cells involved in MBP-induced proliferation was a prerequisite for the generation of the immune response against MBP. Similarly, the promoting effect...
of γδ T cells on dendritic cell maturation also required direct cell-to-cell interaction (45). Importantly, the γδ T cell-assisting effect on the MBP response and EAE transfer was not Ag specific, nor did it require prior contact with Ag. In the reconstitution experiments, regardless whether γδ T cells were derived from MBP-sensitized or naive mice, these cells restored the MBP response and their encephalogenicity. The lack of a requirement for prior Ag stimulation to promote this response suggested that constitutive expression of activation markers on γδ T cells might be instrumental. It has been shown previously that γδ T cells without stimulation express high levels of activation markers (46, 47). The constitutive expression of activation markers by γδ T cells remains to be resolved but may be related to interactions with ubiquitous activation-like receptors. In this regard, it is of interest that γδ T cells can be activated by hsp, and that this activation does not involve APC (20). The expression of hsp is significantly up-regulated in inflammatory tissues, and colocalization of hsp-expressing glial cells and γδ T cells has been documented in MS and EAE (12, 36, 37). It has recently been shown that γδ T cells expressed pattern-type receptors that enable them to interact with other mediators of the innate immune system (35, 48).

An efficient T cell response to Ag requires activation of costimulatory pathways. Lack of a costimulatory signal leads to anergy during primary and probably secondary responses (49). It has also been shown that immune responses can be modulated by several different factors, including regulatory cells, which affect costimulatory pathways (49–51). Depletion of γδ T cells did not influence expression of CD28/CTLA4-B7 and CD40/CD40L molecules on T, B, and CD11b+ cells in LNC responding to MBP. These results suggest that the assisting effect of γδ T cells upon the MBP-specific response was not dependent upon enhanced costimulation. However, when cytokine release by MBP-stimulated LNC depleted of γδ T cells was assessed, significant decreased production of IL-12 was observed. IL-12 is produced exclusively by cells involved in Ag presentation, and its presence determines generation of a Th1-type immune response (52, 53). In agreement with this, IL-12 has been shown to be involved in the regulation of

![Graph of IL-12 production by macrophages](http://www.jimmunol.org/)

**FIGURE 10.** γδ T cells enhance IL-12 production by macrophages in vivo. Mice were injected i.p. with thioglycolate medium to induce a macrophage-rich exudate. After 3 days, animals received PBS i.p., magnetic beads with UC7 or H57 mAbs, or $2 \times 10^6$ γδ or αβ T cells isolated from the spleens of naive mice by magnetic bead separation. On the following day, peritoneal exudate (PE) cells containing >95% macrophages were isolated. Macrophages were cultured for 24 h, and then the culture supernatant was harvested, and IL-12 levels were measured using an ELISA. IL-12 production by macrophages from mice treated with γδ T cells was ~3-fold higher than from control animals ($p < 0.05$).

![Graph of clinical score](http://www.jimmunol.org/)

**FIGURE 11.** The effect of γδ T cells depletion on adoptive transfer EAE can be reversed by addition of IL-12. Recipients ($n = 12$) received $8 \times 10^7$ LNC depleted of γδ T cells by negative selection using the MACS system ( ), or γδ-depleted LNC incubated in vitro with 1 μg of IL-12 for 3 h before cell transfer ( ). Expression of disease was determined according to the clinical scale, as described before. The figure demonstrates the daily mean clinical index (+SD) in both examined groups. Expression of EAE was significantly higher in mice that received LNC stimulated with IL-12 than in mice transferred with γδ-depleted LNC ($p < 0.05$).
several autoimmune diseases, including EAE (52, 54–56). The present γδ T cell reconstitution experiments confirmed that these cells provided a signal for the production of IL-12, and IL-12 added to γδ T cell-depleted LNC restored their proliferative response to MBP and ability to transfer EAE. In additional experiments in vivo, we have proved that γδ T cells may induce IL-12 production by peritoneal macrophages. Similarly, maturation of dendritic cells has been shown to be linked with increased IL-12 release induced by γδ T cells (45). The mechanism underlying γδ T cell-induced IL-12 production by APC remains to be elucidated, but we have shown that the phenomenon requires direct cell-to-cell interaction.

In summary, we provide compelling evidence that γδ T cells positively regulate the expression of EAE during the early induction phase by modulating the function of MBP-reactive encephalitogenic cells. The mechanism involved in this regulation depends upon the induction of IL-12 by APC. Thus, these data should contribute to better understanding of the role of γδ T cells in EAE. This novel interaction between γδ T cells and APC might also provide new targets for immunotherapeutic intervention in autoimmune disorders.

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References


