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Phenotype and Regulation of Persistent Intracerebral T Cells in Murine Toxoplasma Encephalitis

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Toxoplasma gondii is a parasite causing asymptomatic, persistent encephalitis. Protective CD4 and CD8 T cells are recruited to and accumulate in the brain in acute Toxoplasma encephalitis (TE), with slowly decreasing numbers in chronic TE. It is unclear how the size of the intracerebral T cell pool is regulated. Conceivably, permanent recruitment, proliferation, and apoptosis may be involved. We observed that in murine TE recruitment of T cells to the brain was terminated in chronic TE. In vivo 5-bromo-2′-deoxyuridine incorporation and in vitro T cell proliferation experiments revealed that intracerebral T cells did not proliferate, which was explained by the expression of the cell cycle inhibitors p21Waf1/cip1 and p27Kip1 and the inhibitory activity of intracellular FasL. TUNEL staining detected apoptotic T cells at low frequency corresponding to an increased expression of the anti-apoptotic molecules Bcl-2 and Bcl-xl and a reduced expression of the pro-apoptotic molecules Bad, Bax, and Fas ligand in CD4 and CD8 T cells. During progression from acute to chronic TE, both CD4 and CD8 T cells down-regulated CD45RB expression and expressed a differential pattern of cytokines. From these experiments it is concluded that the number of intracerebral T cells increases by recruitment of T cells during acute infection, whereas proliferation of intracerebral T cells does not play a role. In chronic TE, T cell recruitment is terminated, the phenotype of intracerebral T cells changes, and their number is gradually downsized by low level apoptosis, which, however, does not completely resolve the T cell infiltrates. The Journal of Immunology, 2002, 169: 315–322.

O
ral infection of humans and rodents with the obligate intracellular parasite Toxoplasma gondii results in a generalized acute infection. Whereas T. gondii is cleared from most of these organs, the parasite persists in the brain (1). Experimental studies in mice have shown that control of intracerebral (i.c.) T. gondii depends on CD4 and CD8 T cells (2). Although the Ag specificity and epitopes for CD4 and CD8 T cells have not yet been defined, various studies have demonstrated convincingly that i.c. CD4 and CD8 T cells control the parasite mainly by their production of IFN-γ (3, 4). In addition, perforin-mediated lysis of infected cells contributes to control of persisting i.c. toxoplasms (5). In T. gondii-resistant BALB/c mice the balance between the persisting pathogen and i.c. immune reactions results in a chronic latent, clinically asymptomatic encephalitis.

In acute Toxoplasma encephalitis (TE), large numbers of CD4 and CD8 T cells are recruited to the brain (1). In chronic TE, the efficient control of the parasite by CD4 and CD8 T cells results in a decline of the i.c. parasitic load, which is paralleled by decreasing numbers of i.c. T cells. However, some cysts escape the immune response and persist in the brain; therefore, the number of i.c. T cells remains significantly elevated above baseline levels to assure parasite control. The parameters controlling and regulating the size of the i.c. T cell pool have not yet been defined. Several factors, potentially acting in concert may be involved, including continuous recruitment of T cells from the periphery to the CNS, proliferation of i.c. T cells, as well as deletion by apoptosis. Whereas studies of experimental autoimmune encephalomyelitis and most viral infections of the CNS demonstrated that T cells do not proliferate in their target organs (6–9), a few studies provided evidence for an i.c. proliferation of T cells (10–12). In addition, recent studies in murine TE have shown that CD11c⁺ F4/80⁺ cells with phenotypic characteristics of DC can be isolated from the brain and support the ex vivo proliferation of T cells (13). This finding raises the question of whether i.c. T cells proliferate in vivo in TE. Moreover, it is unclear whether apoptosis contributes to the decreasing pool of i.c. T cells in chronic TE. In experimental autoimmune encephalomyelitis massive apoptosis resolves i.c. T cell infiltrates, resulting in termination of the autoimmune attack (14, 15). In addition, in most viral CNS infections T cells rapidly disappear from the brain after elimination of the pathogen, although it has not yet been demonstrated that apoptosis contributes to the resolution of i.c. T cell infiltrates (16).

In general, T cell apoptosis as well as proliferation are regulated by the expression of specific genes. Apoptosis is regulated by several members of the bcl-2 superfamily (17). Bad and bax are pro-apoptotic, whereas bcl-2 and bcl-xL exert anti-apoptotic effects. In addition, engagement of the Fas ligand (FasL) on T cells can induce apoptosis. The proliferation of T cells is controlled by several genes, including p21Waf1/cip1 and p27Kip1, which control progression of the cell cycle from G1 to S phase by inhibiting the activities of cyclin-dependent kinases (18–20).

To address the important issue of how the phenotype of i.c. T cells and their fate are regulated during biphasic, persistent...
infectious encephalitis, TE was analyzed in T. gondii-resistant BALB/c mice. In this experimental model the phenotype, the expression of proliferation- and apoptosis-related genes, as well as the pattern of effector function-associated genes were found to be stage specific and correlated strongly with disease activity. Furthermore, the recruitment of T cells to the T. gondii-infected brain and their apoptotic and proliferative behaviors were differentially regulated during the various stages of the infection, resulting in a finely balanced control of the i.c. T cell pool.

Materials and Methods

Animals

Female BALB/c mice (6–8 wk old) were purchased from Harlan-Winkelmann (Borchen, Germany). All animals were kept under conventional conditions in an isolation facility throughout the experiments.

Parasites and T. gondii infection

RH toxoplasms were grown in vitro in L929 fibroblasts in DMEM supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in 5% CO₂. Parasites were harvested from freshly lysed fibroblasts by centrifuging the tissue culture medium at 50 × g. The supernatant was passed through a 5-μm pore size syringe filter. Thereafter, RH toxoplasms were washed three times in 0.1 M PBS (20 min, 400 × g), counted microscopically, and heat killed at 65°C for 20 min. Heat-killed T. gondii (HKT) were stored at −80°C until use. To infect mice with T. gondii, cysts of the low virulence DX strain of T. gondii were harvested from the brains of chronically infected mice. Brain tissue from these animals was dispersed in 0.1 M PBS (pH 7.4). The final concentration of the infectious agents was adjusted to a dose of five per 0.5 ml, which was applied to the animals by gavage.

Immunohistochemistry and TUNEL staining

Uninfected and T. gondii-infected mice were perfused intracardially with 0.9% saline in deep Metofane (Janssen, Neuss, Germany) anesthesia on the indicated days postinfection (p.i.). Brains of three animals per group were dissected, mounted on thick glass slides, embedded in OCT, and snap-frozen in isopentane (Fluka, Neu-Ulm, Germany) precooled on dry ice, and stored at −80°C. For immunohistochemistry, 10-μm frozen sections were prepared. Immunohistochemistry for detection of apoptotic CD4 and CD8 T cells was performed by application of rat anti-mouse CD4 (clone GK1.5, BD Biosciences, Heidelberg, Germany) and CD8 (clone 53-6.7, BD Biosciences) Abs, respectively, followed by incubation with mouse anti-rat biotin-spacer (Dianova, Hamburg, Germany) and extravadin-FITC (Sigma, Deisenhofen, Germany). Thereafter, the TUNEL kit (Roche, Mannheim, Germany) was applied, according to the manufacturer’s instructions. The number of TUNEL+CD4 and CD8 T cells per square millimeter was semiquantitatively evaluated by use of a grading system: 0 = negative, + = <2% of the leukocyte population positively stained, ++ = 2–10%, +++ = 10–50%, and ++++ = >90%.

Flow cytometric analysis

Cerebral leukocytes were isolated from the brains of anesthetized and perfused mice as described previously (4). In brief, brain tissue was minced through a 100-μm stainless steel sieve, and leukocytes were separated by Percoll gradient centrifugation (Amersham-Pharmacia, Freiburg, Germany). Brain-derived CD4 and CD8 T cells were stained with either rat anti-mouse CD4-FITC (clone GK1.5, BD Biosciences) or rat anti-mouse CD8-FITC (clone 53-6.7, BD Biosciences), respectively, on the indicated days p.i. T cells were costained with rat anti-mouse CD44-PE, CD62 ligand (CD62L; MLLE14)-PE, or CD45RB-PE (all from BD Biosciences). Control staining included incubation of brain-derived leukocytes with unlabeled or fluorochrome-labeled isotype-matched control Abs. Flow cytometry was performed on a FACSscan, and the data were analyzed with CellQuest software (BD Biosciences).

T cell depletion

For detection of CD4 and CD8 T cells, mice were treated with rat anti-mouse CD4 (clone GK1.5; American Type Culture Collection, Manassas, VA), rat anti-mouse CD8 (clone 2.43; American Type Culture Collection), or a combination of these Abs. Abs were purified from tissue culture supernatant of hybridomas by protein G chromatography, adjusted to a concentration of 2.5 mg/ml in 0.1 M PBS, sterile-filtered, and stored at −20°C until used. Control mice were treated with rat IgG (Sigma). Abs were injected i.p. at a concentration of 0.5 mg/ml/mouse at the indicated time points. On the first 3 days of treatment Abs were injected daily. Thereafter, Abs were injected every third day. The efficiency of T cell depletion was controlled by flow cytometry.

5-Bromo-2′-deoxyuridine (BrdU) experiments

The drinking water of uninfected and infected mice (day 36 p.i.) was supplemented with BrdU (2.0 mg/ml; Sigma). Fresh water containing BrdU was prepared daily and mice were treated for 8 days. One day thereafter, leukocytes were isolated from brain and spleen. In addition, on days 14, 15, and 16, respectively, mice were treated i.p. with BrdU (0.8 mg BrdU/mouse in 200 μl PBS). BrdU was applied for 48 h in mice on day 14 p.i., for 24 h in mice from days 15–16 p.i., and for 1 h in mice on day 16 p.i. From these groups of mice leukocytes were isolated from brain and spleen on day 16 p.i. Cells were stained with rat anti-mouse CD4-PE (clone GK1.5; BD Biosciences) or rat anti-mouse CD8-PE (clone 53-6.7; BD Biosciences), fixed and permeabilized with 4% formaldehyde and 0.1% Triton X-100 in 0.1 M PBS, and stained with mouse anti-BrdU-FITC (clone 3D4, BD Biosciences). Control staining was performed with isotype-matched control Abs. Cells were analyzed by flow cytometry with a FACScan (BD Biosciences), and the data were analyzed with CellQuest software (BD Biosciences).

Cell proliferation assays

Splenic leukocytes were isolated by passing spleens through a cell strainer (BD Biosciences), and erythrocytes were lysed by ammonium chloride. Cerebral leukocytes were isolated as described above. Either unseparated leukocytes or selectively isolated brain- or spleen-derived Thy1.2+ T cells or F4/80+ cells, respectively, were used. For the isolation of Thy1.2+ T cells and F4/80+ cells, the MACS system (Miltenyi Biotec, Bergisch-Gladbach, Germany) was applied. In brief, brain- or spleen-derived Thy1.2+ T cells and F4/80+ cells were incubated with rat anti-mouse Thy1.2-FITC (clone 53-2.1; BD Biosciences) or rat anti-mouse F4/80-FITC (clone F4/80; Serotec, Oxford, U.K.), respectively, followed by anti-FITC-coupled paramagnetic beads. Magnetically labeled cells were positively selected. The purity of isolated cell populations was always >95% as controlled by flow cytometry. In proliferation assays using isolated cells, 1 × 10^6 Thy1.2+ T cells and 2 × 10^5 F4/80+ cells were seeded per well. In proliferation assays with bulk leukocytes, 2 × 10^6 cells were used. Cell proliferation assays were performed in 96-well plates, and cells were incubated in MEM-a supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), HEPES (10 mM), L-glutamine (1 mM), 2-ME (10 μM). Triplicate wells were stimulated with HKT (0.4 parasites/cell), Con A (5 μg/ml), or medium. Cells were incubated at 37°C in 5% CO₂. After 72 h, 0.5 Ci [3H]thymidine/100 μl medium was added to the cells. Twenty-four hours thereafter, cells were harvested on glass-fiber plates, and radioactivity was measured with a beta scintillation counter (Beckman, Munich, Germany). Data are presented as counts per minute or as a stimulation index. The stimulation index is defined as the quotient of counts per minute after stimulation with HKT or Con A and counts per minute after stimulation with medium.

Sorting of CD4 and CD8 T cells

To analyze mRNA expression of i.c. CD4 and CD8 T cells, leukocytes were isolated from the brain on days 14 and 30 p.i. Subsequently, cells were stained with rat anti-mouse CD4-PE (clone GK1.5; BD Biosciences) and rat anti-mouse CD8-FITC (clone 53-6.7; BD Biosciences), and CD4+ and CD8+ cells were sorted in 0.1 M PBS (pH 7.4) with a FACSVantage (BD Biosciences). After centrifugation the cell pellet was resuspended in preparation RNA Pure (purchased, Germany) and snap-frozen with 2-methyl-butane precooled on dry ice. Lysed cells were stored at −80°C.

Analysis of CD4 and CD8 T cell mRNA expression by RT-PCR

The expression of cell cycle-associated molecules (p21<sup>Waf1/cip1</sup>, p27<sup>Kip1</sup>), apoptosis-related molecules (Bad, Bax, Bcl-2, Bcl-<i>x</i>), cytokines (IL-2, IL-4, IFN-γ), perforin, and hydroxyphosphorylribosyltransferase was analyzed from sorted i.c. CD4 and CD8 T cells according to a protocol described in detail previously (21). Primer and probe sequences for <i>p21<sup>Waf1/cip1</sup></i>, <i>p27<sup>Kip1</sup></i>, Bad, Bax, Bcl-2, Bcl-<i>x</i>, and perforin are listed in Table 1. In brief, mRNA was extracted from isolated cells (5 × 10^5 sorted CD4+ and CD8+ T cells/extraction, respectively) using an mRNA extraction kit.
After RT of mRNA using the Superscript RT kit (Life Technologies, Eggenstein, Germany), PCR reactions were conducted in a final volume of 10 μl. The PCR reaction conditions were optimized for each set of primers. PCR was performed at different cycle numbers to ensure that amplification occurred in the linear range. PCR products were electrophoresed through an agarose gel, and the DNA was transferred to a nylon membrane (Amersham-Pharmacia). Blots were hybridized using specific oligonucleotide probes, which were 3’-end labeled with digoxigenin using a digoxigenin oligonucleotide 3’-end labeling kit (Roche). A digoxigenin luminescent kit (Roche) was used to visualize the hybridization products.

### Statistical evaluation

For statistical evaluation of cell proliferation assays, Student’s t test was used. A value of p < 0.05 was accepted as significant.

## Results

### Kinetics and phenotype of CD4 and CD8 T cells in TE

The number of i.c. CD4 and CD8 T cells increased from day 0 (noninfected mice) to days 14 and 21 p.i. (acute TE) and declined with development of chronic TE (days 30 and 50 p.i.; Fig. 1). For further detailed analysis days 14 and 30 p.i. were chosen. On day 0 p.i. the number of i.c. T cells was too low to allow a valid analysis of their phenotype.

In TE, i.c. CD4 and CD8 T cells consistently expressed high levels of CD44 and were mostly CD62L negative, which identifies them as activated T cells (Fig. 2). Expression of CD45RB, levels of which are linked to the functional status of T cells, declined on days 14 p.i. the number of i.c. T cells was too low to allow a valid analysis days 14 and 30 p.i. were chosen. On day 0 p.i. the number of i.c. T cells was too low to allow a valid analysis of their phenotype.

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### Analysis of cytokine and perforin mRNA transcription

Analysis of cytokine and perforin mRNA transcription demonstrated that CD4 T cells expressed high levels of IFN-γ, low amounts of IL-4 and IL-2, and low levels of perforin in acute TE (day 14 p.i.; Fig. 3). In chronic TE (day 30 p.i.), CD4 T cells had up-regulated their IL-4 mRNA expression, but down-regulated levels of IFN-γ and perforin mRNA. IL-2 mRNA was only weakly expressed in chronic TE (day 30 p.i.). In i.c. CD8 T cells, the pattern of cytokine and perforin mRNA expression also changed from acute to chronic TE; perforin mRNA expression increased in chronic TE, IFN-γ transcription was largely unchanged at a high level, IL-2 was only weakly transcribed in chronic TE, and IL-4 mRNA was consistently absent. These findings illustrate that both the number and phenotypic characteristics of i.c. T cells changed with the progression of disease.

### Dynamics of T cell recruitment to the T. gondii-infected brain

It has previously been shown that the formation of i.c. T cells infiltrates depends on the recruitment of T cells to the brain in acute TE. In accordance with these findings, application of anti-CD4 and/or anti-CD8 Abs from days 8–14 p.i. resulted in the depletion of the respective T cell subset(s) in both spleen and brain (Fig. 4A). To analyze whether a continuous recruitment of T cells also occurs in chronic TE, CD4 and/or CD8 T cell depletion experiments were performed from days 30–44 p.i. This strategy neither depleted nor reduced the number of CD4 or CD8 T cells in the CNS, whereas the respective T cell subsets were efficiently eliminated from the spleen (Fig. 4B). In addition, at this stage of the disease, T cell depletion experiments did not cause an increase in the i.c. parasitic load (data not shown). These findings indicate that in acute TE the substantial increase in i.c. T cells predominantly results from the recruitment of T cells to the brain, but not from the local expansion of T cells in the brain, which are present in low numbers in the CNS of uninfected mice (Fig. 1). In chronic TE a significant permanent recruitment of T cells did not occur in TE and obviously was not required for control of i.c. persisting parasites.

### Apoptosis of i.c. T cells

To determine whether i.c. T cells undergo apoptosis in TE, TUNEL staining was applied. In acute TE (day 14 p.i.) some TUNEL-positive CD4 and CD8 T cells were detected (+ +), which resided mainly in the meninges and only occasionally in

### Table I. Primer and probe sequences

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<td>p27</td>
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### Figure 1. Kinetics of i.c. CD4 and CD8 T cells.

At each time point, cerebral leukocytes of three mice per experimental group were isolated, counted, stained for CD4 and CD8, and analyzed by flow cytometry. Data represent the mean ± SD from two independent experiments.
perivascular infiltrates (Fig. 5). In chronic TE (day 30 p.i.) only exceptional TUNEL-positive CD4 and CD8 T cells were present (+).

In acute TE, CD4 T cells expressed the pro-apoptotic molecules Bad, Bax, and FasL and lacked expression of the anti-apoptotic molecules Bcl-2 and Bcl-xL (Fig. 6). In chronic TE, CD4 T cells down-regulated expression of Bad, Bax, and FasL, up-regulated Bcl-xL expression, and remained Bcl-2 negative. CD8 T cells strongly expressed Bax mRNA and low amounts of Bad mRNA in both acute and chronic TE. In acute TE, CD8 T cells showed a faint signal for Bcl-2 that was strongly up-regulated in chronic TE. Intracerebral CD8 T cells prominently expressed FasL in both acute and chronic TE. Expression of Bcl-xL was also strongly up-regulated in CD8 T cells during progression from the acute to the chronic stage of TE.

These findings demonstrate that apoptosis contributes to the decline in the number of i.c. T cells. The number of apoptotic i.c. T cells in chronic TE paralleled the reduced expression of pro-apoptotic and the increased expression of anti-apoptotic molecules in CD4 and CD8 T cells, indicating a specific regulation of apoptosis in these two T cell subsets during progression from acute to chronic TE.

BrdU incorporation experiments

In addition to the recruitment of T cells from peripheral lymphatic organs to brain, the proliferation of i.c. T cells may significantly influence the number of i.c. T cells. To analyze whether T cells proliferate in their target organ, BrdU incorporation experiments were performed. As illustrated in Fig. 7, in the normal murine brain the low number of CD4 and CD8 T cells did not incorporate BrdU, and thus did not proliferate. In contrast to those in the brain, CD4 and CD8 T cells in the spleen of uninfected mice incorporated low amounts of BrdU. In acute TE (days 14–16 p.i.), when large numbers of T cells were recruited to the brain, treatment of mice with BrdU resulted in the appearance of BrdU+ cells in both spleen and brain (data not shown). Since i.c. T cells may incorporate BrdU during proliferation in lymphatic organs, the ongoing recruitment of peripheral T cells to the brain precluded a distinction between intra- and extracerebral proliferation. In chronic TE, when T cell recruitment to the brain has terminated, i.c. CD4 and CD8 T cells also did not incorporate BrdU, whereas in spleen the

FIGURE 2. Flow cytometric analysis of CD62L, CD44, and CD45RB expression of i.c. CD4 and CD8 T cells on days 14 and 30 p.i. Cerebral leukocytes were isolated from 6 mice on the respective day p.i., and isolated cells were stained for CD62L, CD44, and CD45RB, respectively, and costained with either anti-CD4 or -CD8. CD4 and CD8 T cells were gated, and histograms of CD62L, CD44, and CD45RB expression are shown. In histograms depicting the expression of rat IgG, CD44, and CD45RB the mean value is included in the upper right corner of the histograms. In histograms illustrating expression of rat IgG and CD62L, a marker region including positive cells is defined, and the percentage of cells in this region is shown. The data from one of three experiments, which yielded similar results, are shown.

FIGURE 3. RT-PCR analysis of IL-2, IL-4, IFN-γ, and perforin mRNA expression in CD4 and CD8 T cells. On days 14 and 30 p.i., i.c. leukocytes were isolated from 6 mice. Leukocytes were stained with CD4 and CD8, and cells were sorted by FACS. For RT-PCR, mRNA isolated from 5 × 10⁵/cell type was used. Data show autoradiograms from one experiment. In a repeat experiment similar data were obtained.

FIGURE 4. T cell depletion experiments. Mice were treated with rat IgG anti-CD4, anti-CD8, or both anti-CD4 and anti-CD8. Initially, mice were treated for 3 consecutive days with 0.5 mg of the respective Ab and every third day thereafter. Mice were treated either from days 8–14 p.i. (A) or from days 30–44 p.i. (B). On day 14 p.i. (A) or 44 p.i. (B) mice were sacrificed, and leukocytes were isolated from brain and spleen, stained for CD4 and CD8 Ag expression, and analyzed by flow cytometry. Each group of mice consisted of six animals, and within one group, leukocytes from brains and spleens, respectively, were pooled and analyzed. Data are presented as dot plots, and the percentage of CD4+ and CD8+ cells is shown. In a second experiment identical data were obtained.
percentage of BrdU+ CD4 and CD8 T cells increased to 7 and 3%, respectively.

At the molecular level, the lack of i.c. T cell proliferation was paralleled by the expression of p21Waf/cip1 mRNA in both CD4 and CD8 T cells throughout TE (Fig. 8). In addition, CD8 T cells strongly expressed p27Kip1 in chronic TE, but not in acute TE. A weak p27Kip1 mRNA expression of CD4 T cells was confined to chronic TE.

These findings indicate that the arrest of i.c. T cell proliferation in the T. gondii-infected brain might result from an intrinsic inhibition of cell cycle progression.

**In vitro proliferation of i.c. T cells**

To determine whether the CNS environment contributes to inhibition of i.c. T cell proliferation, in vitro proliferation assays were performed. In these experiments leukocytes, including T cells and macrophages/microglia, were isolated from the brain and spleen of acutely and chronically infected mice. In both acute and chronic toxoplasmosis, cerebral T cells did not proliferate (Fig. 9). In contrast, splenic T cells proliferated in acute toxoplasmosis in response to Con A, but not to HKT, which is consistent with previously published data (22). In chronic toxoplasmosis, splenic T cells proliferated vigorously in response to both HKT and Con A and had a significantly increased stimulation index compared with i.c. T cells (p < 0.01 for HKT, p < 0.001 for Con A). Moreover,
The inhibition of T cell proliferation may be influenced by two mutually not exclusive factors. First, i.c. T cell may be irreversibly blocked in their proliferation due to factors derived from the microenvironment of the brain. Second, i.c. APCs may be unable to induce the proliferation of T cells or may even actively inhibit T cell proliferation. To address these questions, Thy1.2+ T cells and F4/80+ APCs were isolated from the brain and spleen of chronically infected mice. As shown in Fig. 10, F4/80+ spleen cells efficiently induced the proliferation of Thy1.2+ splenic T cells after restimulation with HKT and Con A. In contrast, brain-derived T cells showed a significantly reduced proliferation rate when F4/80+ spleen cells were used as APC and exhibited a weak response exclusively to HKT, but not to Con A (p < 0.01 for HKT, p < 0.001 for Con A). In addition, the proliferation of spleen-derived T cells was significantly reduced when F4/80+ cells derived from the brain were used instead of spleen-derived APCs (p < 0.005 for HKT, p < 0.01 for Con A). Moreover, splenic T cells exhibited a significantly reduced proliferation rate when a 1/1 mixture of F4/80+ brain- and spleen-derived APCs was used instead of pure splenic APCs (p < 0.01 for HKT, p < 0.01 for Con A).

To analyze whether soluble mediators of i.c. F4/80+ cells inhibit the proliferation of T cells, cerebral leukocytes were isolated from mice on day 30 p.i. and were restimulated with HKT or Con A. The supernatant of these cultures was harvested after 24 h and added to cultures of spleen cells derived from T. gondii-infected mice. The supernatant (final concentration, 20%) did not inhibit the proliferation of splenic T cells induced by restimulation with HKT and Con A (data not shown). In addition, neutralization of IL-10, NO, indoleamine-2,3-dioxygenase, nerve growth factor, TGF-β, and PGE, which have the capacity to suppress T cell proliferation, are known to be produced in the TE or by brain cells in response to T. gondii, did not restore the proliferation of splenic Thy1.2+ T cells when i.c. F4/80+ cells in combination with irradiated splenic feeder cells were used as APCs (data not shown).

In conclusion, these experiments indicate that 1) i.c. T cells were irreversibly blocked in their capacity to proliferate, which could not be overcome by professional splenic APC; 2) i.c. F4/80+ cells actively suppressed T cell proliferation even in the presence of professional splenic APC; and 3) nonsoluble mediators of i.c. F4/80+ suppress T cell proliferation.

**Discussion**

This study addresses the mechanisms that regulate the number and phenotype of i.c. T cells, which are required for the control of i.c.-persisting T. gondii, and correlates these findings with the molecular phenotype of these cells as well as with the regulatory influence of i.c. F4/80+ cells.

Upon acute infection a large number of activated CD4 and CD8 T cells was recruited to the brain. These i.c. T cells did not proliferate in vivo or in vitro, indicating that the recruitment of peripheral T cells to the brain is the driving factor that increases the size of the i.c. T cell pool. In the chronic stage of the infection, depletion of CD4 and/or CD8 T cells for 14 days did not reduce the amount of i.c. T cells, but completely depleted T cells in the spleen. These findings indicate that in contrast to acute TE a substantial ongoing recruitment of T cells from lymphatic organs to the brain does not occur in chronic TE and, moreover, indicates the longevity of i.c. T cells. Since the control of i.c. toxoplasms is strictly T cell dependent (1, 23), these findings also imply that T cells persist in their target organ to mediate protection. In contrast to these findings in T. gondii-resistant BALB/c mice, the combined depletion of CD4 and CD8 T cells in chronic TE of the T. gondii-susceptible C57BL/6 strain results in the depletion of peripheral and i.c. T cells, ultimately leading to a lethal exacerbation of cerebral toxoplasmosis (2). These divergent findings may be explained by a different regulation of T cell recruitment in resistant compared with susceptible mice, but may also be caused by a disturbance of the blood-brain barrier in susceptible animals, allowing access of the depleting Abs to the brain.

In both acute and chronic TE, i.c. T cells did not proliferate, and, interestingly, the nonproliferating phenotype of i.c. T cells could not be reverted in vitro even in the presence of professional APC derived from the spleen, which efficiently supported the proliferation of spleen-derived T cells. The nonproliferating phenotype of i.c. T cells is in agreement with various studies of inflammatory CNS disorders (6–9). The present study demonstrates for the first time that inhibition of proliferation was strongly correlated with the expression of p21Waf1/cip1 and p27Kip1 in i.c. CD4 and CD8 T cells. These two genes decisively control the progression of the cell cycle from G1 to S phase by inhibiting the activities of cyclin-dependent kinases and thereby prevent phosphorylation of the common targets of cyclin-dependent kinases, i.e., the retinoblastoma protein (18–20, 24). The biological importance of p21Waf1/cip1 and p27Kip1 for the inhibition of T cell proliferation in the brain is
further stressed by the observation that in Sindbis virus encephalitis nonproliferating i.c. T cells are characterized by a hypophosphorylated retinoblastoma protein. In infectious diseases, the lack of i.c. T cell proliferation may be functionally important in several aspects. First, an unrestricted increase in i.c. T cells can be prevented that may potentially carry the risk of development of immunopathology, ultimately damaging the highly vulnerable brain in this chronic cerebral infection. Second, i.c. proliferation of T cells in response to Ags presented by i.c. APCs may increase the risk for a sensitization of T cells against brain-derived Ags with the subsequent development of an autoimmune attack. Third, the expression of p21Waf1/cip1 and p27Kip1 and the predicted inhibition of cyclin-dependent kinases may not only prevent proliferation of T cells, but also confer some protection against apoptosis of i.c. T cells, since cyclin-dependent kinases induce apoptosis of nonproliferating T cells (25). The latter assumption is supported by the coincident up-regulation of p21Waf1/cip1 and p27Kip1 and the reduction of the number of apoptotic T cells in chronic TE.

In general, the precise mechanisms leading to the inhibition of i.c. T cell proliferation have not yet been identified, but various i.c. cell populations and factors have the capacity to suppress the proliferation of T cells. In our study i.c. F4/80+ cells of infected animals inhibited the proliferation of cerebral T cells and, moreover, actively suppressed the proliferation of splenic T cells in the presence of splenic APC. This latter observation is in contrast to Sindbis virus encephalitis, an acute, self-limited viral infection of the CNS, in which i.c. leukocytes did not suppress splenic T cell responses (8). Currently, the reason for these divergent findings remains unresolved; however, the observation that i.c. F4/80+ macrophages actively inhibited T cell proliferation via NO production in experimental autoimmune encephalomyelitis (9), a phenomenon that was not observed with TE, indicates a disease-specific regulation of the function of i.c. F4/80+ cells. In TE, i.c. F4/80+ cells are a heterogeneous cell population composed of microglia and macrophages (4). In addition, recent studies have identified macrophages/microglia expressing CD11c, an Ag that is preferentially expressed on DC (13). Remarkably, although 1) both i.c. macrophages and microglia express MHC class I and II Ags as well as costimulatory molecules, including B7-1, B7-2, and LFA-1 (1, 13), and, even more important, 2) selectively isolated CD11c+ macrophages/microglia from the T. gondii-infected brain supported the proliferation of T cells in vitro (13), the bulk of i.c. F4/80+ cells inhibited T cell proliferation. In the present study neutralization of iNOS, IL-10, PGE, TGF-β, nerve growth factor, and indoleamine-2,3-dioxygenase, all of which may suppress the proliferation of T cells and which are produced in response to T. gondii by resident brain cells (4, 22, 26–30), did not reverse the inhibitory activity of i.c. F4/80+ cells. In addition, the supernatant of restimulated cerebral leukocytes did not suppress proliferation of splenic leukocytes. Collectively, these findings argue for an inhibition of i.c. T cell proliferation by as yet undefined cell surface molecules of i.c. F4/80+ cells.

The decline of the pool of i.c. T cells was a rather slow process, and the low level of i.c. T cell apoptosis may well be sufficient to induce this process. The low level of T cell apoptosis is remarkable, because previous studies in experimental autoimmune encephalomyelitis have shown that autoreactive T cells are eliminated within a few days from the brain via apoptosis (14, 15) and that in viral CNS infections T cells are rapidly eliminated from the brain after eradication of the virus (16). These divergent findings clearly illustrate that in a chronic cerebral infection, in which T cells are indispensable for control of the pathogen, apoptosis is partially blocked. In TE, both CD4 and CD8 T cells expressed pro- and anti-apoptotic molecules, and in general, both T cell populations exhibited a reduced expression of the pro-apoptotic molecule Bad, Bax, and FasL, but showed up-regulated expression of the anti-apoptotic molecules Bcl-2 and Bcl-xL, which can inhibit Bad-, Bax-, and FasL-induced apoptosis during the progression from acute to chronic TE. These kinetics correlate well with the low level of apoptosis in chronic TE. Factors other than mRNA levels of the aforementioned molecules, including the loss of mitochondrial membrane potential, reactive oxygen species, phosphorylation, and dimerization of bcl-2 family members may also influence the apoptosis of cells (17). The functional importance of anti-apoptotic mechanisms in chronic toxoplasmosis is strongly supported by studies in NF-κB-deficient mice. These mice succumbed to a chronic TE characterized by a increased rate of apoptosis of splenic T cells with an increased expression of Fas and FasL on these cells (31). Although it is unknown whether these mice had lost their i.c. T cells caused by an overshooting rate of apoptosis or whether splenic T cells underwent apoptosis due to the absence of DC, these findings strongly support the assumption that apoptosis-inhibiting pathways are crucial to prevent elimination of i.c. T cell in chronic TE and loss of immune control in the CNS.

During progression from acute to chronic TE, both i.c. CD4 and CD8 T cells down-regulated the expression of CD45RB, a molecule with a reduced expression on memory T cells as well as on Th2 cells compared with Th1 cells (32, 33). In accordance with these data, down-regulation of CD45RB Ag was accompanied by reduced IFN-γ and increased IL-4 mRNA expression of i.c. CD4 T cells as well as increased perforin transcription, but a constantly high IFN-γ transcription of CD8 T cells. The increased expression of IL-4 by CD4 T cells and of perforin by CD8 T cells in chronic TE indicates that these molecules are of importance for the i.c. anti-parasitic immune response in the chronic stage of infection. In fact, both IL-4- and perforin-deficient mice have a diminished capacity to control i.c. T. gondii and succumb to necrotizing TE in the chronic phase of infection (5, 34).

In conclusion, the present study indicates that in T. gondii-resistant mice the immune system controls i.c. toxoplasmosis with the lowest possible expenditure concerning T cell turnover. The results of this study are also of importance for understanding of the mechanisms underlying reactivation of latent TE in AIDS patients, implying that loss of i.c. T cells will result in an insufficient number of protective i.c. T cells, which cannot be substituted for by proliferation of T cells in their target organ or by a continuous recruitment of T cells to the brain.

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