RESEARCH ARTICLE

Regulation of Microglia by CD4+ and CD8+ T Cells: Selective Analysis in CD45-congenic Normal and *Toxoplasma gondii*-infected Bone Marrow Chimeras

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Microglia, the resident macrophage population of the central nervous system, is rapidly activated in murine *Toxoplasma* encephalitis (TE). However, the precise contribution of microglia to intracerebral immune reactions and the *in vivo* regulation of microglial activity are still poorly understood. To selectively analyse microglial reactions in TE, we have established a model of radiation-induced CD45-congenic bone marrow chimeras between CD45.2+ C57BL/6 (recipient) and CD45.1+ B6.SJL (donor) mice. These chimeras allow a differentiation of radioresistant CD45.2+ microglia from all other leukocytes, which exhibit the CD45.1+ haplotype. In the normal brain, microglia produced tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-10, and IL-15 mRNA. In TE, marked microglial activation was observed with a *de novo* expression of IL-12p40 and inducible nitric oxide synthase mRNA, upregulation of IL-1β and TNF-α mRNA, a continuous production of IL-10, and IL-15 mRNA, an induction of major histocompatibility class I and II antigens, intercellular adhesion molecule-1, and leukocyte function-associated antigen-1. Furthermore, selective depletion of CD4+ and/or CD8+ T cells in the chimeras revealed that microglial cytokine production was critically regulated by CD8+ T cells, whereas expression of cell surface molecules was less dependent on T cells. These findings demonstrate a specific regulation of microglia by T lymphocytes during the course of TE.

Introduction

Toxoplasma (T.) gondii is an obligate intracellular parasite which persists in the host brain. Reactivation of latent intracerebral (i.c.) toxoplasms is prevented by a continuous T cell response. Impairment of the anti-parasitic T cell response in immunodeficient patients may result in the development of a reactivated *Toxoplasma encephalitis* (TE). Indeed, immunodeficient patients, especially those with AIDS, are at high risk for opportunistic TE (28).

In experimental TE, the i.c. immune response is characterized by the recruitment of inflammatory leukocytes including CD4+ and CD8+ T cells to the brain (35). Moreover, microglia, the resident macrophage population of the central nervous system (CNS), is activated and expresses various immunologically important cell surface molecules (6). There is also evidence that microglia contributes to the i.c. immune response by the production of cytokines, but the selective cytokine production of microglia has so far not been characterized. Recent *in vivo* studies illustrate that the activation of microglia in TE is critically dependent on interferon (IFN)-γ receptor-, but not on tumor necrosis factor (TNF) receptor 1 or 2-mediated immune responses (10). Since CD4+ and CD8+ T cells are the cellular sources of IFN-γ in TE (36), it is highly suggestive that T cells are the major regulator of microglia in TE. However, the relative importance of the two T cell populations to microglial cell activation is still unknown and may be distinct.

A selective analysis of microglia is hampered by the lack of a selective microglia marker which would reliably distinguish microglia from other leukocytes, in particular macrophages. To overcome the difficulty in the selective isolation and analysis of microglia in the adult brain, we took advantage of the facts that *1*. microglia expresses lower levels of CD45 than other leukocytes

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(38), *2.* microglia is radioresistant in contrast to other leukocytes and their precursors (21, 22), and *3.* co-isogenic CD45 mouse strains are available, which exhibit either the CD45.1 or CD45.2 haplotype. In order to establish an experimental model in which microglia is defined by a stable genetic marker, we generated bone marrow chimeras using CD45.1+ B6.SJL-*Ptprc^a Pep3^b*/BoyJ (Ly5.1) mice as donors and CD45.2+ C57BL/6 mice as recipients (designated throughout as B6.SJL and B6 mice, respectively). In this model we addressed the regulation of microglia by CD4+ and CD8+ T cells in TE.

Material and Methods

Animals. Female C57BL/6 (CD45.2, H-2^b, abbreviated as B6) and NMRI mice (both 8 to 10 weeks old) were purchased from Harlan-Winkelmann (Borchen, Germany). Breeding pairs of B6.SJL-*Ptprc^aPep3^b*/ BoyJ (Ly5.1, CD45.1, H-2^b, designated as B6.SJL) mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). B6.SJL mice were bred under specific pathogen free-conditions in flexible film isolators. Female B6.SJL mice, less than 6 months old, were used as donor for bone marrow transplantation.

Bone marrow chimeras. For the generation of bone marrow chimeras, B6 mice were irradiated (1000 rad). Within 2h thereafter, B6 mice were intravenously reconstituted with 2×10^7 bone marrow cells isolated from the tibia and femur of B6 or B6.SJL mice to generate B6→B6 and B6.SJL→B6 bone marrow chimeras, respectively. The drinking water was supplemented with neomycin sulfate (Sigma, Deisenhofen, Germany) for two weeks. Ten weeks after bone marrow transplantation the composition of blood leukocyte populations from B6, B6→B6 and B6.SJL→B6 mice was assessed by flow cytometry. After lysis of erythrocytes with ammoniumchloride, leukocytes obtained from the retroorbital plexus were stained with phycoerythrin (PE)-labeled CD45.1 and fluorescein isothiocyanate (FITC)-conjugated CD45.2 (Pharmingen, Hamburg, Germany) or with anti-CD4, anti-CD8, anti-B220, anti-F4/80, and anti-Ly6G followed by goat anti-rat-PE (Biozol, Freising, Germany), and CD45.1-FITC or CD45.2-FITC, respectively.

Infection. The ME49 strain of *T. gondii* (kindly provided by Dr. W. Bohne, University of Würzburg, Germany) was used for infection. *T. gondii* cysts were harvested from the brains of chronically infected NMRI mice. Isolated cysts were suspended in sterile phosphate-buffered saline (PBS, pH 7.4) and adjusted to a concentration of 10 cysts / 500 μl PBS, which was injected i.p. into B6 mice, B6→B6 or B6.SJL→B6 chimeras at 10 to 12 weeks after bone marrow transplantation.

T cell depletion experiments. T. gondii-infected B6.SJL→B6 chimeras were depleted of CD4+ and CD8+ T cell subsets by intraperitoneal injection of 0.5 mg anti-CD4 (clone GK 1.5, rat IgG_{2a}), 0.5 mg anti-CD8 (clone 2.43, rat IgG_{2b}), or both antibodies for 3 successive days with additional injections every third day. The antibodies had been purified from tissue culture supernatants by affinity chromatography using HiTrap protein G columns (Amersham-Pharmacia, Freiburg, Germany). Control mice were inoculated with an identical dose of rat IgG (Sigma). Treatment was initiated at the indicated days post infection (p.i.). The effect of CD4+ and CD8+ T cell depletion was confirmed by flow cytometry of spleen and brain cells using anti-CD4 and anti-CD8 antibodies.

Histopathological analysis. Brains were prepared from deeply anesthetized mice intracardially perfused with 0.9% NaCl, mounted on thick filter paper with O.T.C. compound (Miles Scientific, Naperville, IL), snap-frozen in isopentane preecooled with dry ice, and stored at –80°C until use. CD4+ and CD8+ T cells, B220+ B cells, Ly6G+ granulocytes, F4/80+ macrophages/ microglia as well as major histocompatibility (MHC) class I and II antigens, leukocyte function-associated antigen (LFA)-1, intercellular cell adhesion molecule (ICAM)-1, and *Toxoplasma* antigen were demonstrated immunohistochemically on cryostat sections as described previously (6, 9, 35). In brief, for identification of *T. gondii*, CD4, CD8, and B220 antigens, an indirect peroxidase protocol using goat anti-rabbit or sheep anti-rat peroxidase labelled F(ab)_2′ fragments (Pharmingen, Amersham-Pharmacia), respectively, were used. All other antigens including CD45.1 and CD45.2 were detected by the application of biotin-labelled CD45.1 or CD45.2 (Pharmingen) followed by peroxidase-conjugated avidin/biotin complex (Dakopatts, Hamburg, Germany). Peroxidase reaction product was visualized using 3,3′-diaminobenzidine tetrahydrochloride (Sigma) as substrate. Sections were in part lightly counterstained with hemalum.

Flow cytometry. For the analysis of microglia and inflammatory i.c. leukocytes, brain tissue of perfused mice was mechanically disrupted, and leukocytes were
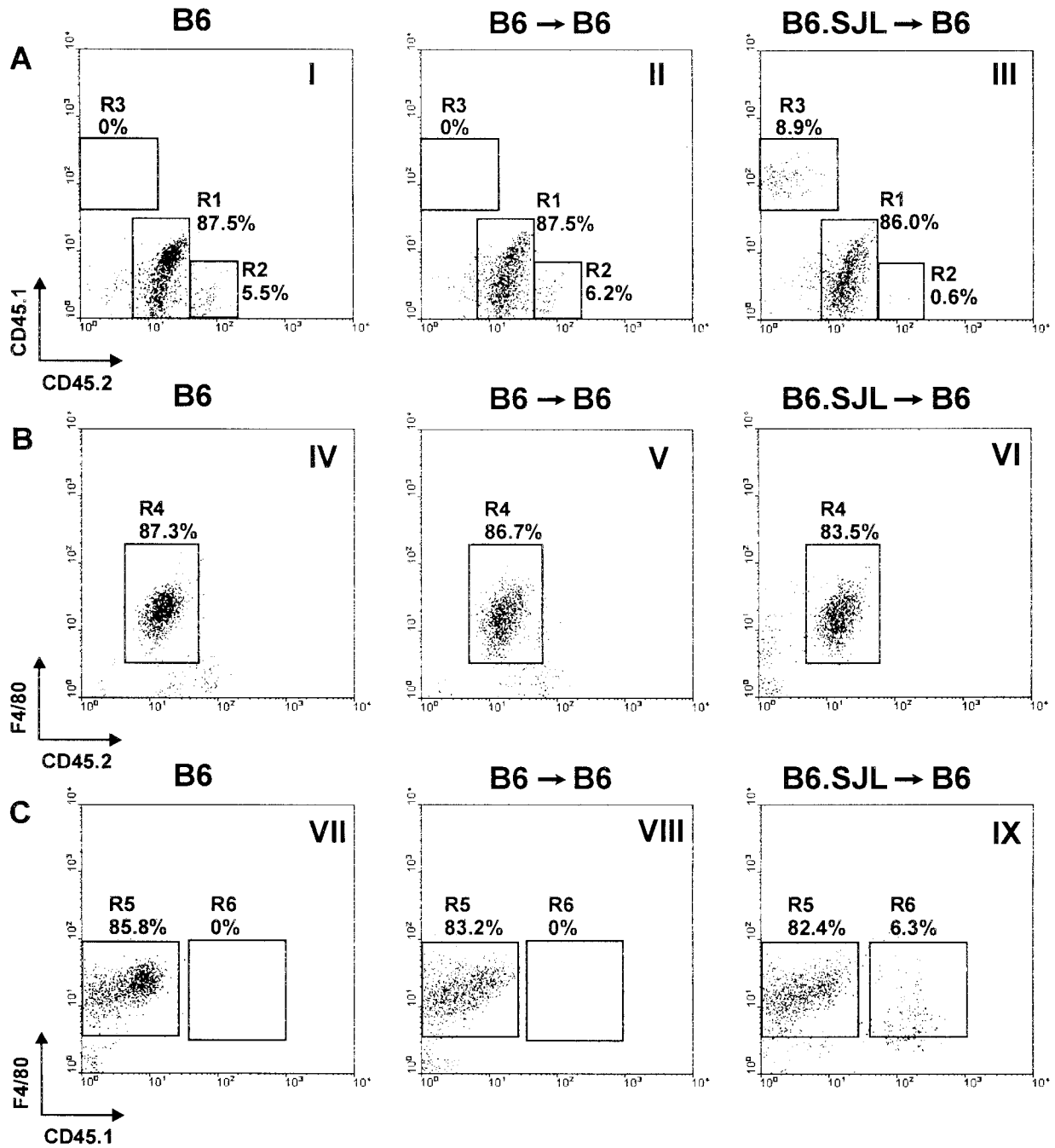
Figure 1. Flow cytometric characterization of CD45$^+$ leukocytes in the brain of normal B6 (**A**, I; **B**, IV; **C**, VII), B6→B6 (**A**, II; **B**, V; **C**, VIII), and B6.SJL→B6 (**A**, III; **B**, VI; **C**, IX) mice.

Leukocytes were isolated from the brains of uninfected B6, B6→B6, and B6.SJL→B6 mice by mechanical disruption of brain tissue followed by density gradient centrifugation 12 weeks after bone marrow transplantation. Isolated leukocytes of six mice per group were pooled and the percentage of CD45.1$^+$ and CD45.2$^+$ leukocytes was assessed by flow cytometry. R1 depicts CD45.2^{low}, R2 CD45.2^{high}, R3 CD45.1$^+$, R4 F4/80$^+$CD45.2^{low}, R5 F4/80$^+$CD45.1$^-$, and R6 F4/80$^+$CD45.1$^+$ cells. The percentage of cells in each region is shown.
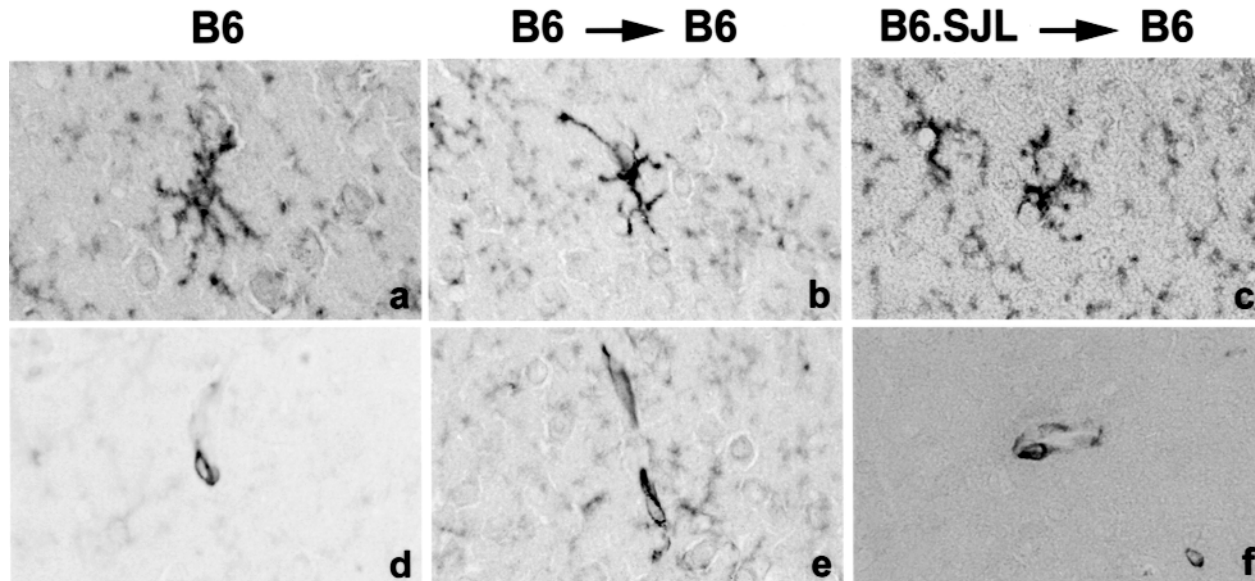
Figure 2. Phenotypic profile of microglia and perivascular cells in the brains of normal B6 mice (**a**, **d**), B6→B6 (**b**, **e**) and B6.SJL→B6 (**c**, **f**) bone marrow chimeras.

In B6 mice and B6→B6 chimeras, microglia (**a**, **b**) with finely branched, short, thin processes as well as perivascular cells (**d**, **e**) are CD45.2⁺. In contrast, in B6.SJL→B6 chimeras, only microglia is CD45.2⁺, whereas perivascular cells are CD45.1⁺. **a-e**: Anti-CD45.2 immunostaining, **f**: anti-CD45.1 immunostaining. **a**, **b**, **d-f**: ×550, **c**: ×500.

separated by density gradient centrifugation as described previously (10, 36). The composition of isolated cells was assessed in B6, B6→B6, and B6.SJL→B6 mice by staining with either CD45.1-PE and CD45.2-FITC, anti-F4/80-PE (Camon, Wiesbaden, Germany) and CD45.2-FITC, or anti-F4/80-PE and CD45.1-FITC. In B6.SJL→B6 chimeras the activation of F4/80⁺ CD45.2⁺ microglia and the phenotypic composition of CD45.1⁺ inflammatory cells were analysed by co-staining for MHC class I, MHC class II, LFA-1, and ICAM-1 antigens or CD4, CD8, B220, F4/80, and Ly6G, respectively, in accordance to protocols described earlier (6, 35). Flow cytometry was performed on a FACScan (Becton Dickinson).

Selective isolation of microglia. For the separation of microglia from other leukocytes, isolated i.c. cells of B6.SJL→B6 chimeras were incubated with CD45.2-FITC followed by paramagnetic anti-FITC microbeads (Milteny, Bergisch-Gladbach, Germany). Subsequently, cells were passed over a MACS separation column (Milteny) and the CD45.2⁺ fraction was collected. To determine the percentage of CD45.2⁺ F4/80⁺ microglial cells before and after separation, 1×10^5 cells before and 5×10^4 cells after MACS were additionally stained with F4/80-PE and analysed by flow cytometry.

Detection of cytokine mRNA by RT–PCR. The following cytokine mRNAs were analysed in isolated microglial cells by RT-PCR as reported previously (9, 10): interleukin (IL)-1β, IL-10, IL-12p40, IL-15, TNF-α, IFN-γ, iNOS, and hydroxyphosphoribosyltransferase (HPRT). Primer and probe sequences for IL-12p40 and IL-15 were as follows: IL-12p40, 5′-GTGAAGCAC-CAAATTACTCCGG-3′ (sense), 5′-GCTTCATCTG-CAAGTTCTTGGG-3′ (antisense), 5′-CAGTGTCCT-GCCAGGAGGATGT-3′ (probe); IL-15, 5′-GTTCTCT TCTTCATCCTCCC-3′ (sense), 5′-GTGTTCTTAAG-GACCTCACC-3′ (antisense), 5′-CTTGCAGTGCATC TCCTTAC-3′ (probe). For mRNA isolation, 1.5×10^5 microglial cells were used. In addition, IFN-γ and HPRT mRNA expression was determined in brain tissue homogenates. In brief, mRNA was extracted from either brain tissue homogenates or isolated microglia by use of an mRNA extraction kit (Amersham-Pharmacia). After reverse transcription of mRNA using the Superscript RT Kit (Life Technologies, Eggenstein, Germany), PCR were carried out in a volume of 10-30 μl. PCR conditions were optimized for each set of primers to ensure that amplification occurred in the linear range. PCR products were subjected to electrophoresis through an agarose gel, and the DNA was transferred to a nylon membrane (Amersham-Pharmacia). Blots were hybridized using specific oligonucleotide probes, which
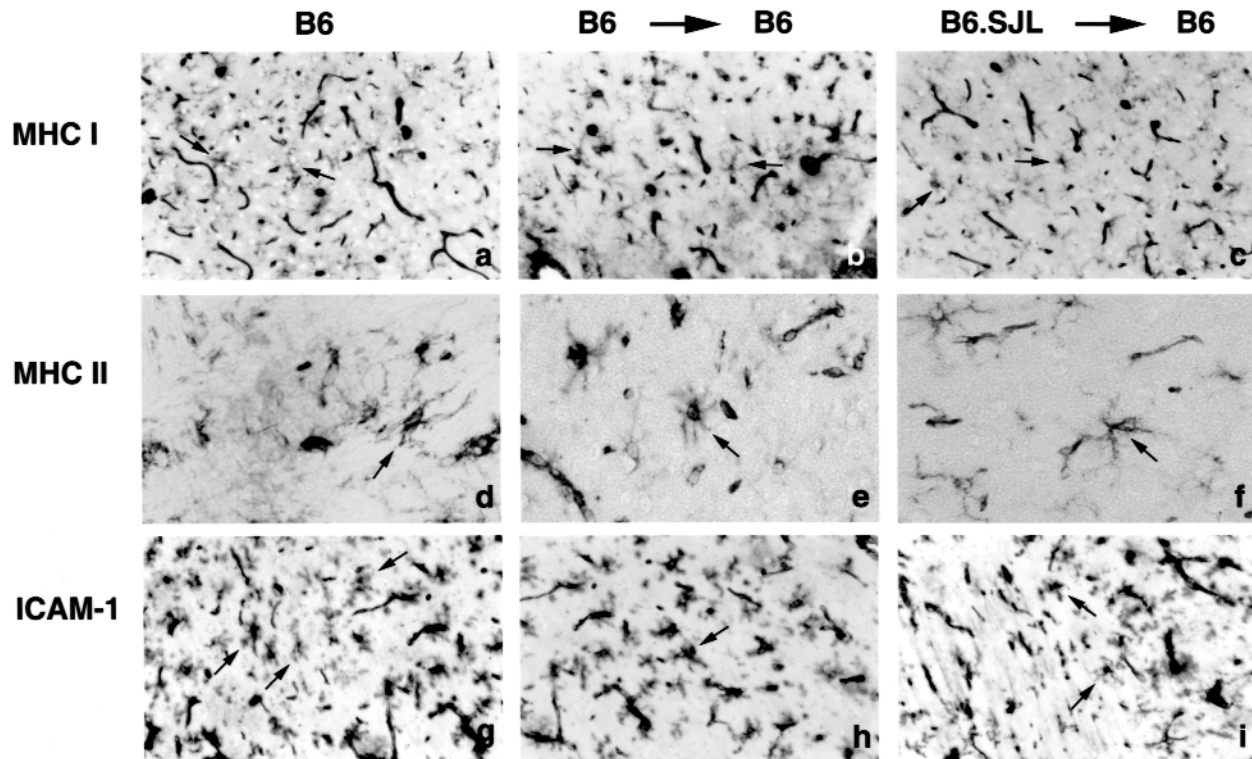
Figure 3. Normal activation of microglia in B6 mice (**a**, **d**, **g**), B6→B6 (**b**, **e**, **h**) and B6.SJL→B6 (**c**, **f**, **i**) chimeras. At day 13 p.i., microglia was activated in all experimental groups as evidenced by induction of MHC class I antigens (**a–c**), MHC class II antigens (**d–f**), and ICAM-1 (**g–i**, arrows). **a–c**: Anti-H-2 immunostaining, ×170; **d–f**: anti-I-A immunostaining; **d-f**: ×330; **g-i**: anti-ICAM-1 immunostaining, ×170.

were 3′-end labeled with digoxigenin by use of a DIG labeling kit (Roche Diagnostics, Mannheim, Germany). A DIG luminescent kit (Roche Diagnostics) was used to visualize the hybridization products.

Statistics. For statistical evaluation of the i.c. parasitic load, the number of parasites was determined on anti-*T. gondii* immunostained sections in the various experimental groups. High power fields (HPF, n = 100-400) were analysed per section in three animals from each group on days 7 and 13 p.i. Data are presented as mean ± SD. The statistical significance was analysed by use of the Student's *t* test. A *p* value < 0.05 was accepted as significant. Experiments were performed twice.

Results

Phenotype of B6.SJL (CD45.1)→B6 (CD45.2) bone marrow chimeras. Combined flow cytometry of i.c. leukocytes and immunohistochemistry were applied to precisely characterize the phenotype of bone marrow chimeras. In the normal brain, FACS analysis identified

~87.3% of i.c. leukocytes as F4/80⁺ microglial cells, which were characterized by a weak co-expression of the CD45.2 antigen (CD45.2ᴸᵒʷ, Figure 1, Panel A, I, R1; Figure 1, Panel B, IV, R4; Figure 2a). In addition, 5.5% CD45.2ʰⁱᵍʰ leukocytes were detected (Figure 1, Panel A, I, R2), which corresponded in part to F4/80⁺ plexus macrophages and perivascular macrophages (Figure 2d) as revealed by immunohistochemistry. B6→B6 bone marrow chimeras showed an identical phenotypic composition of i.c. leukocytes (Figure 1, Panel A, II; Figure 1, Panel B, V; Figure 2b,e), thus demonstrating that irradiation and injection of congenic bone marrow does not affect the phenotype of leukocytes in the brain.

In CD45.1⁺ B6.SJL→CD45.2⁺ B6 bone marrow chimeras microglial cells retained their CD45.2ᴸᵒʷ phenotype as evidenced by FACS (Figure 1, Panel A, III; Figure 2c). In leukocyte preparations from uninfected CD45.1⁺ B6.SJL→CD45.2⁺ B6 bone marrow chimeras the vast majority (~84.0%) of cerebral leukocytes are microglia as shown by their co-expression of the CD45.2ᴸᵒʷ and F4/80 antigens (Figure 1, Panel B, VI, R4). Thus, the combined expression of these cell surface
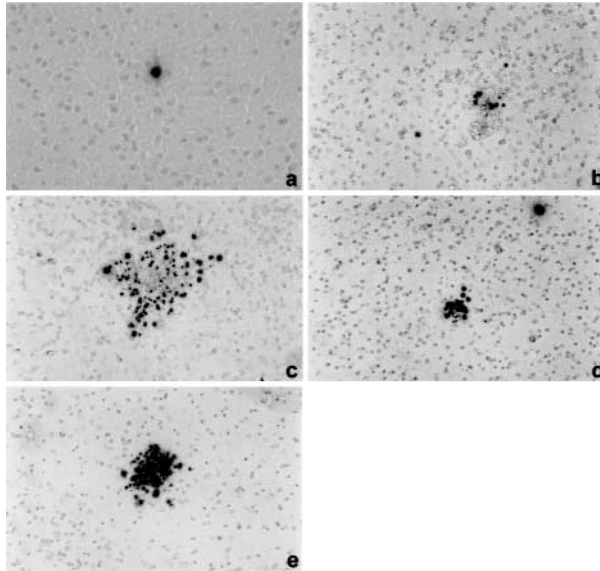
Figure 4. Distribution of *T. gondii* antigen in the brains of B6.SJL→ B6 chimeras in TE.

a. At day 7 p.i., single parasites are detectable in the brain tissue.

b. At day 13 p.i., the number of i.c. parasites has increased in rat IgG treated mice. Small clusters of *T. gondii* antigen are present in the vicinity of a blood vessel.

c. At day 13 p.i., a large necrotic focus is decorated by numerous parasites in an anti-CD8 treated mouse.

d. In an anti-CD4 treated mouse, small clusters of *T. gondii* antigen are present at day 13 p.i. Note the absence of parasite-associated necrosis.

e. Large clusters of *T gondii* antigen with a central necrosis are present in a mouse which had received a combined anti-CD4 and anti-CD8 treatment at day 13 p.i.

a-e. Anti-*T. gondii* immunostaining, slight counterstaining with hemalum. **a**: ×100, **b–d**: ×60.
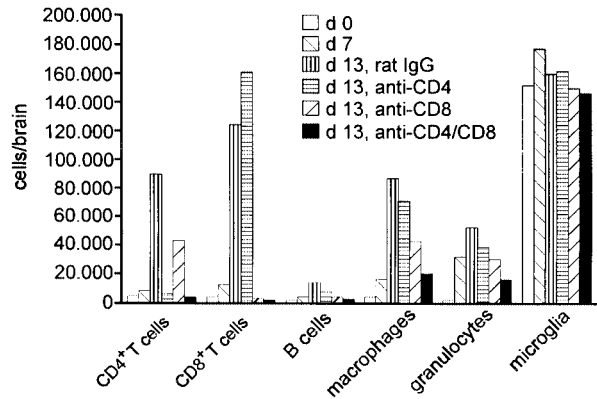

Figure 5. Phenotypic composition of i.c. leukocytes in normal and *T. gondii*-infected B6.SJL→B6 chimeras.
Leukocytes were isolated from the brains of uninfected (d 0) and infected (d 7 and d 13 p.i.) B6.SJL→B6 chimeras by mechanical disruption of brain tissue followed by density gradient centrifugation. Isolated leukocytes of six mice per group were pooled, and the composition of i.c. leukocytes was assessed by flow cytometry. Data represent the mean of six mice per group. For depletion of CD4^{+} and/or CD8^{+} T cells treatment with the respective antibodies was performed from day 7 to 13 p.i.. In a separate experiment similar results were obtained.

molecules and their levels of CD45.2 expression (38) identified the isolated population as microglia. Furthermore, CD45.1^{+} cells in the brain were entirely CD45.1[high] (Figure 1, Panel A, III, R3). Co-staining for CD45.1 and F4/80 revealed that these CD45.1^{high} cells were mostly F4/80[+] (Figure 1, Panel C, IX, R6), identifying them as inflammatory macrophages. Immunohistochemical staining of serial sections identified these CD45.1^{+} cells as plexus macrophages and perivascular cells (Figure 2f). In contrast, CD45.1^{+} cells with the morphological characteristics of microglia, i.e. cells with a small, rod shaped nucleus and short, thin cellular processes, were not detected *in situ* (Figure 2f). In addition, CD45.1^{low} cells were not identified in the brains of normal CD45.1[+] B6.SJL→CD45.2[+] B6 bone marrow chimeras by flow cytometry. Taken together, these data indicate that there was no evidence for a differentiation of CD45.1[+] donor-derived leukocytes into microglia.

As a further control, peripheral blood leukocytes from CD45.1[+] B6.SJL→CD45.2[+] B6 bone marrow chimeras were analysed for the state of chimerism. Flow cytometry demonstrated that CD45.1[+] bone marrow cells effectively replaced hematogenous host cells and that all monocytes/macrophages, granuolcytes, B cells, and virtually all T cells (purity in excess of 95% with only 2-3% contamination: 95 ± 2% for CD4^{+} T cells and 96 ± 3 % for CD8^{+} T cells) have converted to the CD45.1^{+} donor phenotype ten weeks after transplantation.

Microglia of B6.SJLÆB6 chimeras shows a normal activation pattern in TE. To analyse whether irradiation and transplantation of congenic CD45.1[+] bone marrow influenced the capacity of microglia to respond to infectious stimuli, microglial reactions were evaluated by immunohistochemistry and flow cytometry in normal and *T. gondii*-infected B6, B6→B6, and B6.SJL→B6 mice. Whereas MHC class I and II antigens as well as ICAM-1 were absent on microglia in uninfected mice, infection with *T. gondii* induced a strong upregulation of these cell surface molecules throughout the brain in all experimental groups at day 13 p.i. (Figures 3, 7). There was no evidence for regional differences in microglial activation. These findings illustrate that in bone marrow chimeras microglia retains its immunologically down-
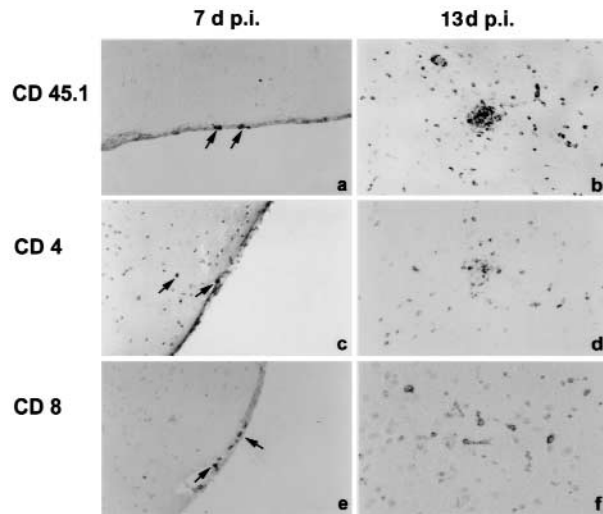
Figure 6. Recruitment of CD45.1⁺ leukocytes to the brains of *T. gondii*-infected B6.SJL→B6 chimeras.

a. At day 7 p.i., some CD45.1⁺ leukocytes are present in the leptomeninges. CD45.1 immunostaining, slight counterstaining with hemalum, ×60.

b. From day 7 to 13 p.i., the number of CD45.1⁺ leukocytes in the brain has markedly increased, and inflammatory infiltrates are present in the brain parenchyma and in association with blood vessels. Anti-CD45.1 immunostaining, slight counterstaining with hemalum, ×60.

c. At day 7 p.i., single CD4⁺ T lymphocytes adhere to the meninges and have already invaded the brain parenchyma. Anti-CD4 immunostaining, slight counterstaining with hemalum, ×60.

d. At day 13 p.i., CD4⁺ T lymphocytes are part of the inflammatory infiltrates or are scattered throughout the brain. Anti-CD4 immunostaining, slight counterstaining with hemalum, ×60.

e. CD8⁺ T lymphocytes are present in the leptomeninges at day 7 p.i. Anti-CD8 immunostaining, slight counterstaining with hemalum, ×60.

f. At day 13 p.i., CD8⁺ T cells are present in the brain tissue. Anti-CD8 immunostaining, slight counterstaining with hemalum, ×60.

regulated phenotype in the normal brain and that microglial cell activation is unimpaired in TE.

Control of i.c. toxoplasms by CD4⁺ and CD8⁺ T cells. In agreement with published data (10, 35), parasites had gained access to the brain as early as day 7 p.i., and up to day 13 p.i. the number of i.c. parasites rised significantly B6.SJL→B6 mice (2.3 ± 1.2 *T. gondii*/100 HPF at day 7 p.i., 73.2 ± 15.3 *T. gondii* /100 HPF at day 13 p.i., p < 0.01, Figure 4a, b). Infection resulted in a steadily increasing recruitment of inflammatory leukocytes to the brain. The i.c. infiltrates were mainly composed of CD45.1⁺ CD4⁺ and CD8⁺ T cells and, to a lesser extent, of CD45.1⁺ F4/80⁺ macrophages, CD45.1⁺
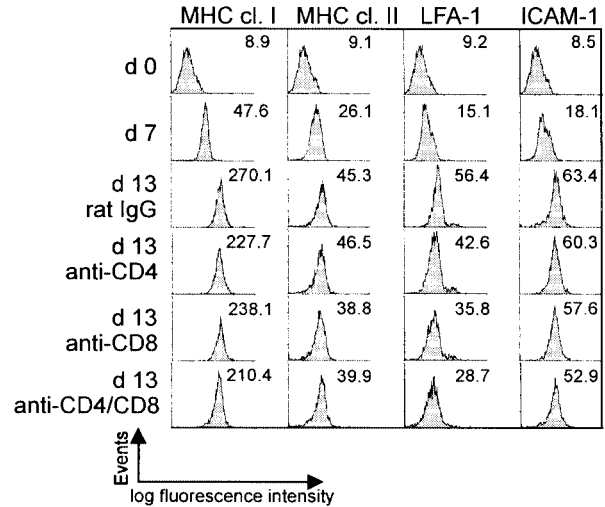

Figure 7. Expression of MHC class I and II antigens, LFA-1, and ICAM-1 on microglia of and *T. gondii*-infected B6.SJL→B6 chimeras.

I.c. leukocytes were isolated from the brains of six mice per group and leukocytes of each group were pooled. Histograms illustrate the expression of MHC class I and II, LFA-1, and ICAM-1 antigens on microglial cells identified by co-staining for CD45.2 and F4/80. The mean fluorescence intensity is shown in each histogram. In control stainings with isotype-matched control antibodies the mean fluorescence intensity was always below 10 (data not shown). In T cell depleted animals, depletion was initiated at day 7 p.i. and continued up to day 13 p.i.

Ly6G⁺, F4/80⁻ granulocytes, and CD45.1⁺ B220⁺ B cells (Figures 5, 6).

Application of anti-CD4 and/or anti-CD8 antibodies from day 7, when infiltration of T cells into the parasite-infected brain is just initiated, to day 13 p.i. highly efficiently reduced the respective T cell subsets in the brain (Figure 5, > 95% reduction for CD4⁺ and CD8⁺ T cells, respectively). Interestingly, depletion of CD4⁺ T cells was accompanied by a compensatory increase of i.c. CD8⁺ T cells, whereas depletion of CD8⁺ T cells resulted in a concomitant decline of i.c. CD4⁺ T cells (Figure 5). These cross-regulatory effects were confined to the brain and did not occur in the spleen as revealed by flow cytometry (data not shown). Depletion of CD8⁺ T cells resulted in a substantial increase of the i.c. parasitic load (118.5 ± 24.2 *T. gondii* /100 HPF) at day 13 p.i., whereas depletion of CD4⁺ T cells caused only a slight increase in the number of i.c. toxoplasms (84.9 ± 15.9 *T. gondii* /100 HPF) at day 13 p.i. as compared to control mice (Figure 4b-d). The highest i.c. parasitic load was detected in mice depleted of both CD4⁺ and CD8⁺ T cells at day 13 p.i. (198.8 ± 29.7 *T. gondii* /100 HPF; p < 0.05 vs. control antibody treated mice, Figure 4e).
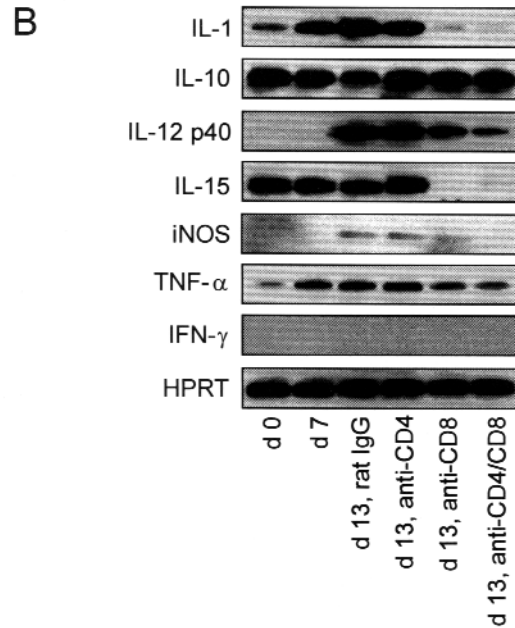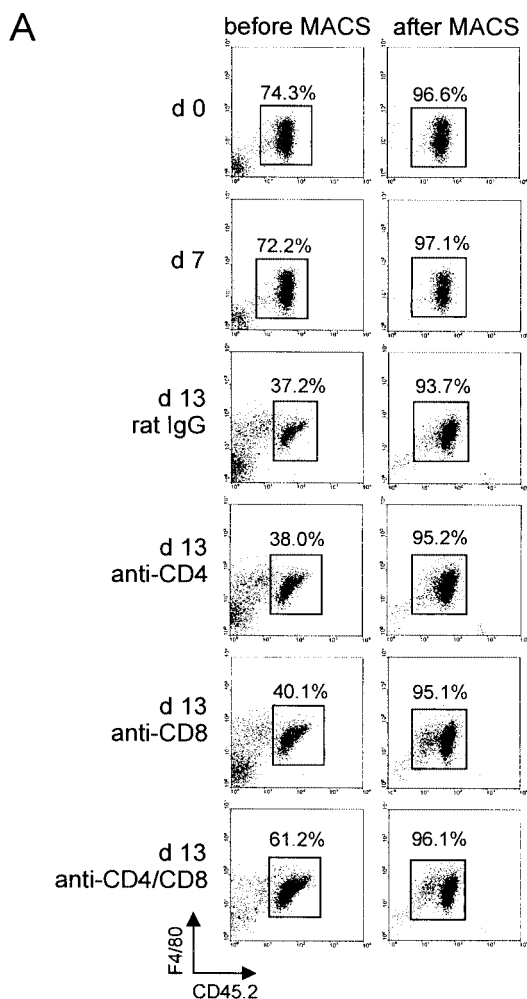
Figure 8. A. Dot plots of i.c. leukocytes stained for CD45.2 and F4/80 before and after MACS. CD45.2⁺ F4/80⁺ microglial cells are marked, and the percentage of these cells is shown. Brains of eight to 12 mice per experimental group were pooled for MACS isolation of CD45.2⁺ cells. **B.** RT-PCR analysis of cytokine mRNA expression in MACS-isolated CD45.2⁺ cells. In each experimental group, 1.5×10^5 cells were used for mRNA extraction.

Thus, i.c. toxoplasms are controlled by both CD4⁺ and CD8⁺ T cells, but CD8⁺ T cells appeared to be slightly more important than CD4⁺ T cells.

Regulation of microglial cell activation and cytokine production by CD4⁺ and CD8⁺ T cells in TE. To assess the activation and regulation of microglial cells by T cells in TE the expression of immunologically important cell surface molecules was evaluated by flow cytometry and immunohistochemistry in uninfected and anti-CD4 and/or anti-CD8-treated *T. gondii*-infected B6.SJL→B6 chimeras (Figure 7). *T. gondii* infection gradually induced MHC class I and II antigens, ICAM-1, and LFA-1 on CD45.2⁺ F4/80⁺ microglia up to day 13 p.i. Depletion of CD4⁺ and/or CD8⁺ T cells starting at day 7 p.i. resulted in a slight decline of MHC class I and LFA-1, whereas MHC class II and ICAM-1

expression were only marginally affected. Immunohistochemical analysis confirmed these findings and showed the same kinetics of MHC class I and II, LFA-1, and ICAM-1 expression on microglial cells without any topographical differences.

To analyse microglial cytokine production and its regulation by CD4⁺ and CD8⁺ T cells, CD45.2⁺ microglia of uninfected and *T. gondii*-infected B6.SJL→B6 chimeras was isolated by MACS. As illustrated in Figure 8A, 93.7%-97.1% of the isolated CD45.2⁺ cells were F4/80⁺ and therefore, microglia. PCR analysis of cytokine mRNA transcripts of isolated microglia revealed already low levels of IL-1β, TNF-α, IL-10 and IL-15 mRNA, but not of IL-12 p40 and iNOS mRNA in uninfected animals (Figure 8B). At day 7 p.i., microglia upregulated IL-1β and TNF-α mRNA and continued to produce IL-10 and IL-15 mRNA. Up to
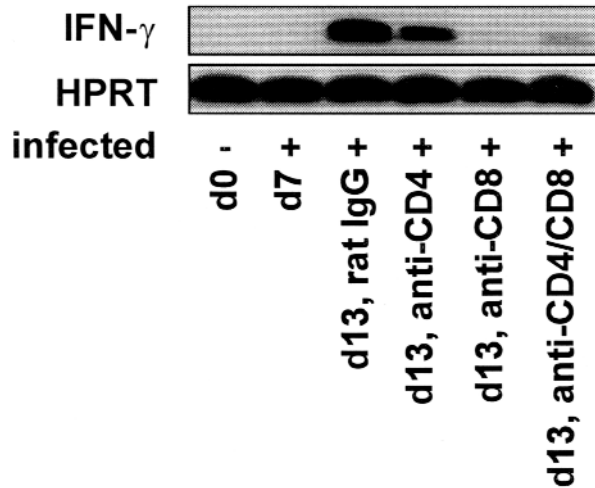
IFN-γ
HPRT
infected

d0 -
d7 +
d13, rat IgG +
d13, anti-CD4 +
d13, anti-CD8 +
d13, anti-CD4/CD8 +

Figure 9. RT-PCR analysis of IFN-γ mRNA expression in brain tissue homogenates.
At the indicated time points 3 mice per experimental group were analysed and representative autoradiograms are shown.

day 13 p.i., IL-1β mRNA levels further increased and a *de novo* expression of IL-12p40 and iNOS mRNA was observed, whereas IL-10, IL-15, and TNF-α mRNA expression was largely unchanged.

Depletion of CD4+ T cells initiated at day 7 p.i. slightly reduced iNOS mRNA expression but did not influence mRNA production of other cytokines by microglial cells, whereas depletion of CD8+ T cells markedly reduced IL-1β, IL-12p40, IL-15, iNOS and TNF-α mRNA transcripts, but not IL-10 mRNA production. Simultaneous depletion of both CD4+ and CD8+ T cells resulted in a further decline of IL-1β, TNF-α, IL-12p40, and iNOS mRNA expression, whereas the expression of the other cytokines was comparable to CD8+ T cell depleted mice. Repeated RT-PCR analysis demonstrated that IFN-γ mRNA transcripts could not be detected in isolated microglia in any of the experimental groups, thereby further confirming the purity of the isolated microglia cell fraction. Taken together, these findings demonstrate that CD8+ T cells, but not CD4+ T cells are the major regulator of microglial cytokine production in TE.

Influence of CD4+ and CD8+ T cell depletion on i.c. IFN-γ production. To analyse whether either CD4+ or CD8+ T cells, both of which produce IFN-γ in TE of BALB/c mice (36), are the major source of this cytokine in *T. gondii*-infected B6.SJL→B6 chimeras, T cell depletion experiments were performed. Depletion of either CD8+ or both CD4+ and CD8+ T cells resulted in

an almost complete loss of IFN-γ mRNA production in brain tissue homogenates (Figure 9). In contrast, depletion of CD4+ T cells caused only a slight reduction of i.c. IFN-γ mRNA levels. Thus, CD8+ T cells, in addition to being the major regulator of microglial cytokine production, are also the major source of IFN-γ in B6.SJL→B6 chimeras.

Discussion

The present study demonstrates that microglia actively participates in the i.c. anti-*T. gondii* immune response by the expression of immunologically important cell surface molecules as well as by the production of cytokines. This activation of microglia in TE is decisively regulated by T cells and, in particular, CD8+ T cells are the major regulator of microglial cytokine production.

The results of the present study were made possible by the use of murine radiation-induced CD45-congenic bone marrow chimeras, which allow a selective characterization of microglial reactions *in vivo*. The suitability of congenic bone marrow chimeras for the precise analysis of microglia has originally been shown in MHC congenic rat chimeras, which were established to study the origin and turnover of microglia and other bone-marrow-derived cell populations in the brain (21, 22). In the present study, we used magnetically isolated CD45.2+ microglia from CD45.1→CD45.2 bone marrow chimeras. This technique allows the selective isolation of microglia to a high degree of purity as evidenced by flow cytometry, which demonstrated *1.* that in CD45.1+ B6.SJL→CD45.2+ B6 bone marrow chimeras CD45.2+ cells are almost entirely microglia (Figure 1, 2), *2.* that all CD45.2high macrophages in the brain were replaced by donor cells (Figures 1, 2), and *3.* that the amount of residual CD45.2+ F4/80- cells was very low (Figure 8A). The purity of the isolated microglia was further stressed by the consistent absence of IFN-γ mRNA in repeated RT-PCR analyses (Figure 8B). Thus, MACS provided the opportunity to systematically analyze a broad spectrum of microglia-derived cytokines in the normal and *T. gondii*-infected murine brain and to evaluate their regulation by CD4+ and CD8+ T cells. Previous studies with FACS-sorted microglia had been successfully applied to demonstrate production of either TNF-α or IL-12p40 in the brains of mice with experimental autoimmune encephalomyelitis (EAE) (25, 33); however, a systematic characterization of a broader spectrum of microglial cytokines in the normal and inflamed brain has not yet been performed.

Interestingly, in the normal brain, microglia

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expressed a remarkable panel of cytokine mRNA including IL-1β, TNF-α, IL-10, and IL-15. These findings extend our recent analysis of cytokine production of F4/80^{+} cells isolated from the brains of normal BALB/c mice, which represent a mixture of predominantly microglia and a minor fraction of macrophages (36). This pattern of microglial cytokines in the normal brain is in contrast to reports from unstimulated cultured microglia, which, in general, does not produce significant levels of cytokines (14). However, cultured microglia may not adequately reflect the *in vivo* situation, since neighboring brain parenchymal cells including neurons and astrocytes, which form a permanently active, integral neuroimmune network together with microglia, are absent. In addition, cultured microglia is generally derived from neonatal mice and, thus, represents a mixture of microglia and macrophages. Furthermore, these cells change in culture under the hospitability of FCS and other supplements during a 1-2 week maturation process, and, most important, functionally, microglia directly isolated from the adult murine brain differed significantly from cultured neonatal microglia in their capacity to present antigen (2). In the normal brain, the function of microglia derived cytokines is still unknown and may not be confined to immunological, but may also include neurophysiological as well as neuroprotective activities (29, 31, 32).

In TE, microglia upregulated IL-1β, TNF-α, and IL-15 mRNA production, exhibited a *de novo* expression of IL-12p40 and iNOS and continued to produce IL-10 mRNA. This spectrum of cytokines is also synthesized by *T. gondii*-infected IFN-γ stimulated murine peritoneal macrophages *in vitro* (11, 19, 27) indicating that in toxoplasmosis brain-resident macrophages, i.e. microglia, show a cytokine pattern comparable to other macrophage populations. In fact, *in vitro* studies have disclosed that IFN-γ activated microglia produces TNF and reactive nitrogen intermediates upon infection with *T. gondii* (4, 5, 12), but production of other cytokines by cultured microglia has so far not been addressed. Previously, the microglia-derived cytokines identified in the present study have been shown to be protective in toxoplasmosis either by the regulation and augmentation of the anti-parasitic immune response (IL-10, IL-12p40, IL-15, iNOS) or — in part simultaneously — by a direct anti-parasitic activity (IL-1β, TNF-α, iNOS) (3, 8, 17, 18, 20, 23, 24, 34). Thus, in TE cytokines produced by microglia may be involved both in parasite control as well as in the regulation of the i.c. immune response. A potential anti-parasitic function of microglia is supported by *in vitro* studies which illustrated that microglial cells are toxoplasmastatic after stimulation with IFN-γ and TNF-α (4, 5).

The suggested immunoregulatory role of microglia is further corroborated by the ubiquitous induction of MHC class I and II molecules in combination with ICAM-1 and LFA-1 on microglia in TE (6, this study). The expression of these cell surface molecules, which were absent from microglia in the normal brain, potentially enables microglia to directly interact with both CD4^{+} and CD8^{+} T cells. However, it remains to be shown if the protective activity of i.c. T cells requires the presence of MHC molecules and cell adhesion molecules on microglia. Such a regulatory role has recently been proposed for microglia in EAE of rat CD45-congenic bone marrow chimeras (13). With respect to persisting toxoplasms in humans, an impairment or dysregulation of these outlined microglial activities by infection of microglia with HIV (1, 26) might contribute to the reactivation of latent toxoplasms in AIDS patients.

A major finding of the present work relates to the regulation of microglial responses by T lymphocytes. Especially, microglial cytokine production was dependent on T cells. Animals depleted of both CD4^{+} and CD8^{+} T cells exhibited a marked decline of microglial IL-1β, IL-12p40, IL-15, iNOS and TNF-α mRNA levels. In contrast, production of IL-10, a potent immunosuppressive cytokine, which inhibits killing of toxoplasms by IFN-γ-activated macrophages (16), was not reduced in these animals. The decline of the aforementioned cytokine mRNA transcripts in CD4^{+} plus CD8^{+} T cell depleted mice is most probably explained by the almost complete reduction of i.c. IFN-γ as revealed by RT-PCR of brain tissue homogenates. This assumption is corroborated by the observation that IFN-γ, the major cytokine protecting against *T. gondii* (7, 39), being produced by both CD4^{+} and CD8^{+} T cells but not microglial cells in TE (36), has a strong capacity to activate microglia in this disorder (10). The combined absence of T cells, IFN-γ, and microglial-derived protective cytokines resulted in a significant increase of the i.c. parasitic load in CD4^{+} plus CD8^{+} T cell depleted mice. This is in agreement with previous reports in chronic TE, which demonstrated, first, a lethal reactivation of TE either by the simultaneous depletion of CD4^{+} and CD8^{+} T cells (15) or by the neutralization of IFN-γ (40), and, second, a downregulation of macrophage/microglial activation due to lack of IFN-γ (18).

Depletion of CD8^{+} T cells, but not of CD4^{+} T cells, also resulted in a marked decrease of cytokine production by microglia illustrating that CD8^{+} T cells are the major regulator of microglial cytokine production in TE.

We assume that this difference reflects the compensatory increase of CD8^{+} T cells in mice depleted of CD4^{+} T cells, whereas depletion of CD8^{+} T cell was accompanied by a decline of CD4^{+} T cells. Although the underlying mechanism of this cross-regulatory effect remains unclear, it resulted in strongly reduced IFN-γ mRNA levels in the brains of CD8^{+} T cell depleted mice as compared to CD4^{+} T cell depleted animals. Consequently, CD8^{+} T cell depleted mice had a higher i.c. parasitic load as compared to CD4^{+} T cell depleted mice, although this difference did not reach statistical significance.

In conclusion, the combination of radiation induced CD45 bone marrow chimeras and MACS permitted the selective analysis of microglial reactions *in vivo*. The data of the present study perform a rationale to selectively explore the functional role of individual microglia-derived cytokines and cell surface molecules in the neuroimmune network protecting against *T. gondii*. Such open questions could elegantly be addressed in bone marrow chimeras between relevant knock out mice and wild type strains, a strategy which should potentially also allow a more precise characterization of the *in vivo* microglial cell responses in other inflammatory as well as non-inflammatory disorders.

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