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Spontaneous inflammatory demyelinating disease in transgenic mice showing central nervous system-specific expression of tumor necrosis factor α

(autoimmunity/disease model/neuroimmunology/inflammatory/cytokine)

LESLEY PROBERT*, KATERINA AKASSOGLOU*, MANOLIS PASPARAKIS*, GEORGE KONTOGEORGOS†, AND GEORGE KOLLIAS*‡

*Department of Molecular Genetics, Hellenic Pasteur Institute, 127, Vas. Sofias Avenue, 115 21 Athens, Greece; and †Department of Pathology, General Hospital of Athens, 154, Messogion Avenue, 115 27 Athens, Greece

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ABSTRACT **Cytokines are now recognized to play important roles in the physiology of the central nervous system (CNS) during health and disease. Tumor necrosis factor α (TNF-α) has been implicated in the pathogenesis of several human CNS disorders including multiple sclerosis, AIDS dementia, and cerebral malaria. We have generated transgenic mice that constitutively express a murine TNF-α transgene, under the control of its own promoter, specifically in their CNS and that spontaneously develop a chronic inflammatory demyelinating disease with 100% penetrance from around 3–8 weeks of age. High-level expression of the transgene was seen in neurons distributed throughout the brain. Disease is manifested by ataxia, seizures, and paresis and leads to early death. Histopathological analysis revealed infiltration of the meninges and CNS parenchyma by CD4$^+$ and CD8$^+$ T lymphocytes, widespread reactive astrocytosis and microgliosis, and focal demyelination. The direct action of TNF-α in the pathogenesis of this disease was confirmed by peripheral administration of a neutralizing anti-murine TNF-α antibody. This treatment completely prevented the development of neurological symptoms, T-cell infiltration into the CNS parenchyma, astrocytosis, and demyelination, and greatly reduced the severity of reactive microgliosis. These results demonstrate that overexpression of TNF-α in the CNS can cause abnormalities in nervous system structure and function. The disease induced in TNF-α transgenic mice shows clinical and histopathological features characteristic of inflammatory demyelinating CNS disorders in humans, and these mice represent a relevant *in vivo* model for their further study.**

The nervous system and the immune system show an intimate structural and functional relationship and exhibit extensive bidirectional communication, which is facilitated by the use of common regulatory factors and receptors (1, 2). Cytokines, classically associated with the modulation of immune function and inflammatory response, are now also emerging as important mediators of central nervous system (CNS) function (3). Tumor necrosis factor α (TNF-α) is a powerful proinflammatory cytokine (for review, see ref. 4), which is produced by neurons in the normal murine brain (5) and exerts neuromodulatory effects (6). The expression of TNF-α is upregulated in a wide range of CNS disorders including multiple sclerosis (MS) (7, 8), AIDS dementia complex (9), bacterial meningitis (10), Parkinson disease (11), and human and murine cerebral malaria (12). This increased expression might derive from activated infiltrating T cells or macrophages (4), activated astrocytes (13), and microglial cells (14). The contribution of increased TNF-α production in the CNS to disease pathogenesis and its mechanism of action are at present ill-defined. However, there is strong evidence that in the CNS, TNF-α acts as a potent proinflammatory cytokine and a major effector of immune-mediated demyelination (15, 16).

In our studies we have been addressing the role of TNF-α *in vivo* using transgenic mice. In one line, which expresses a modified 3′-untranslated region murine TNF-α transgene under the control of its own promoter, we have obtained an expression pattern specific to the CNS. From between 3 and 8 weeks of age, mice spontaneously develop a chronic demyelinating disease, which is manifested by progressive neurological disturbances and leads to premature death within ≈8 months. Our data establish TNF-α as a major effector molecule in chronic inflammatory demyelination in the CNS. The transgenic line described in this study represents an important model for gaining further insight into the pathogenesis of similar diseases in humans.

MATERIALS AND METHODS

Preparation of the Murine TNF-α–Globin Gene Construct and Generation of Transgenic Mice. The murine TNF-α gene was derived from a 129 SV mouse genomic library (Stratagene) using an *Eco*RI–*Nar* I fragment from the 5′ end of the murine TNF-α gene (kindly provided by C. V. Jongeneel, Ludwig Institute for Cancer Research, Lausanne, Switzerland) as a probe. The murine TNF-α–globin gene construct (see Fig. 1*A*) was prepared in the pBluescript vector (Stratagene) as follows: the 2.8-kb *Eco*RI fragment, which contains the promoter and entire coding region of the murine TNF-α gene, was ligated to the 0.77-kb *Eco*RI–*Pst* I/*Sal* I fragment, which contains the 3′-untranslated region and polyadenylylation site of the human β-globin gene (17). A *Bam*HI–*Sal* I fragment was microinjected into fertilized eggs of (CBA × C57BL/6)F_1 hybrid mice as described (18, 19). Colonies of mice were maintained in both conventional and specific-pathogen-free facilities at the Hellenic Pasteur Institute.

RNA Preparation and Analysis. Total RNA was extracted from freshly dissected mouse tissues and cell isolates using the lithium chloride/urea method (20). Peritoneal macrophages were prepared as described (21). Splenocytes were incubated in RPMI containing 5% (vol/vol) fetal calf serum with or without the addition of concanavalin A (Sigma) at 1 μg/ml and phorbol 12-myristate 13-acetate (Sigma) at 10 μg/ml for 8 hr at 37°C in 5% CO_2/95% air. Astrocyte cultures were prepared as described elsewhere (22). S1 nuclease protection analysis

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Abbreviations: CNS, central nervous system; TNF-α, tumor necrosis factor α; GFAP, glial fibrillary acidic protein; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; P, postnatal day.

‡To whom reprint requests should be addressed.

using 5′ murine TNF-α, 3′ β-globin, and human β-actin DNA probes was carried out using standard procedures (21, 23).

In situ hybridization was performed essentially according to Wilkinson *et al.* (24) using single-stranded ^{35}S-labeled murine TNF-α and β-globin RNA probes on parasagittal sections taken from 4% (wt/vol) paraformaldehyde-fixed, Paraplast (BDH)-embedded brains of Tg6074 mice and normal control littermates.

Histology and Immunocytochemistry. Histological analysis was carried out using standard hematoxylin/eosin, oil red/hematoxylin, and luxol fast blue/cresyl violet staining procedures. Immunocytochemistry was carried out using the Vectastain ABC alkaline phosphatase method (Vector Laboratories) as described (21). Antibodies to the following markers were used: glial fibrillary acidic protein (GFAP; astrocyte marker; Dakopatts, Glostrup, Denmark), F4/80 (microglial and macrophage marker; Serotec), CD18 (microglia and leukocytes; provided by Leslie Moloney, Tanabe Research Laboratories, San Diego), CD4 and CD8 (mature T lymphocytes; YTS191.1.2 and YTS169.4.2, respectively; provided by Steve Cobbold, University of Oxford, United Kingdom).

TNF-α Monoclonal Antibody Injections. Transgenic mice (n = 9) received, from birth, once-weekly intraperitoneal injections of a chimeric hamster/mouse monoclonal antibody to murine TNF-α bearing the variable regions of the hamster TN3.19.12 antibody (20 μg/g of body weight in saline) (provided by Roly Foulkes, Celltech, Berkshire, U.K.), whereas transgenic (n = 5) and normal (n = 15) littermates remained untreated or received an equivalent concentration of control hamster IgG (Pierce) (n = 5 transgenic mice). Animal weights and clinical signs were recorded weekly for 11 consecutive weeks.

Measurement of Serum Murine TNF-α. Serum levels of murine TNF-α protein were measured by ELISA (Endogen, Cambridge, MA) according to the manufacturer's specifications.

RESULTS

Murine TNF-α Transgenic Mice. Earlier transgenic studies in our laboratory showed that replacement of the 3′-untranslated region and 3′-flanking DNA sequences of a human TNF-α transgene with similar sequences from the human β-globin gene resulted in its constitutive overexpression in several mouse tissues (25). Microinjection of a similar murine TNF-α–human β-globin hybrid gene construct (Fig. 1*A*) into the pronuclei of (CBA × C57BL/6)F_2 zygotes resulted in seven transgenic founder lines. The transgenic progeny of one founder (Tg6074) develop severe neurological disturbances with 100% phenotypic penetrance and die prematurely, within ≈8 months. The transgene copy number in heterozygous Tg6074 progeny was estimated to be >100 copies per cell by Southern hybridization analysis. Due to the severity of the phenotype, maintenance of the line is achieved by the administration of a neutralizing anti-murine TNF-α monoclonal antibody (see *Materials and Methods*).

Expression of the Transgene in Tg6074 Mice Is CNS Specific. S1 nuclease protection analysis of RNA from Tg6074 tissues demonstrated that the transgene is expressed specifically in the CNS (Fig. 1*B*). Correct initiation and termination of transcription from the transgene were confirmed using 5′ murine TNF-α and 3′ human β-globin S1 probes, respectively. Analysis of CNS RNA from tissues of Tg6074 mice using a 3′ murine TNF-α S1 probe, which specifically measures endogenous TNF-α expression, demonstrated that low levels of endogenous TNF-α mRNA are also produced (data not shown). Analysis of macroscopically dissected regions of the CNS showed transgene expression in all areas tested—cerebral cortex, cerebellum, diencephalon, brain stem, and spinal cord (Fig. 1*B*). *In situ* hybridization carried out on parasagittal sections taken from adult mice showed transgene expression to be localized in neuron cell bodies (Fig. 2). Positive neurons are distributed throughout the brain and are particularly numerous in the thalamus (Fig. 2*A*), entorhinal cortex (Fig. 2*C*), pons, and hypothalamus. Astrocyte cultures taken from Tg6074 whole brain showed no detectable transgene expression by S1 nuclease protection analysis (data not shown). Moreover, it is unlikely that CNS expression is mediated by infiltrating inflammatory cells since transgene expression was not detectable in activated peritoneal macrophages (Fig. 1*C*) or activated splenocytes (data not shown).

We have studied the developmental time course of transgene expression by S1 nuclease protection analysis of RNA, taken from the whole brain of mice, at ages ranging from embryonic day 14 to postnatal day 75 (P75). Expression was first detectable at low levels in P6 mice and increased greatly in intensity until P15 after which expression remained at high levels (Fig. 1*C*).

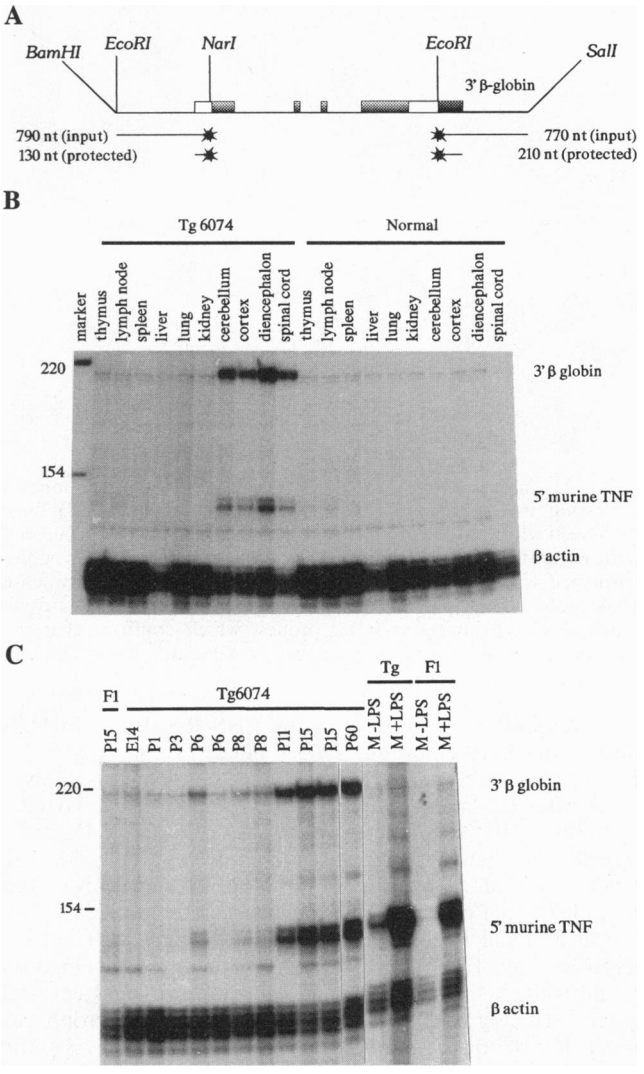

FIG. 1. (*A*) Structure of the murine TNF-α–globin construct that was microinjected into mouse zygotes. The 5′ murine TNF-α and 3′ β-globin DNA probes used for S1 nuclease protection analysis are also represented. (*B*) S1 nuclease protection analysis of total RNA from normal F_1 and Tg6074 tissues. (*C*) S1 nuclease protection analysis of whole brains of normal F_1 and Tg6074 embryos (embryonic day 14; E14), postnatal mice (P1–P60), and thioglycollate-elicited peritoneal macrophages (M) before (−) and after (+) induction by lipopolysaccharide (LPS). Twenty-five micrograms of total RNA was hybridized to a 790-nt 5′ ^{32}P-end-labeled *Eco*RI–*Nar* I probe from the 5′ end of the murine TNF-α gene and to a 770-nt 3′ ^{32}P-end-labeled *Eco*RI–Sal I probe derived from the 3′ end of the human β-globin gene (26).

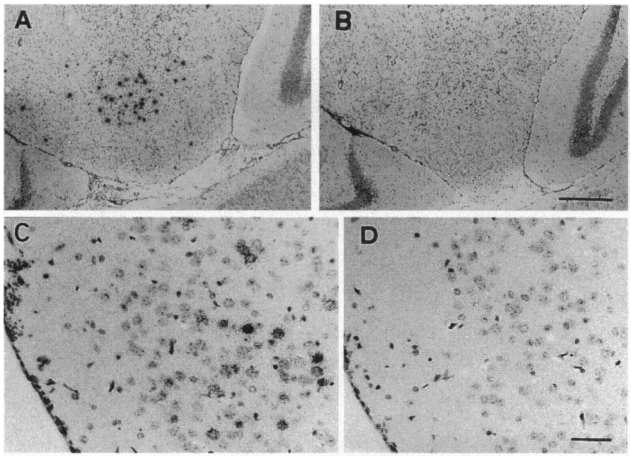

FIG. 2. *In situ* hybridization analysis of TNF-α–globin mRNA expression in the thalamus of Tg6074 (*A*) and normal (*B*) mice hybridized with ^{35}S-labeled murine TNF-α antisense RNA probe and in the entorhinal cortex of a Tg6074 animal showing neuron cell bodies hybridized with the same probe (*C*) or the corresponding control sense RNA probe (*D*). A parallel localization pattern was observed using human β-globin antisense RNA probes, which confirms that the observed expression was transgene specific. (*A* and *B*, bar = 100 μm; *C* and *D*, bar = 25 μm.)

Overexpression of TNF-α in the CNS Results in Inflammation and Demyelination. Tg6074 mice develop progressive disease characterized by random seizures, ataxia, and imbalances, often leading to hind limb paresis and kyphosis. To date, mortality is 100% by 8 months of age (n = 326). Death is typically associated with paralysis and wasting (Fig. 3). The development of disease did not differ between animals reared in conventional or specific-pathogen-free conditions.

Conventional histopathological analysis of CNS from severely affected Tg6074 mice revealed extensive demyelination in the white matter of the medulla oblongata and cervical spinal cord (Fig. 4*A*) and numerous lipid-laden macrophages and mylein debris in the inner white and molecular layers of the cerebellum (Fig. 4*C*). There was no histological evidence of systemic disease in peripheral tissues. Immunocytochemistry revealed further changes characteristic of CNS inflammation and pathology. Reactive astrocytosis and microgliosis were seen throughout the brain and spinal cord of sick animals by using antibodies to GFAP (Fig. 4*D*) and F4/80 or CD18 (Fig. 4*E*), respectively. In addition, large numbers of CD4- (Fig. 4*F*) and CD8- (Fig. 4*G*) immunoreactive T cells were found in the subarachnoid space and infiltrating the CNS parenchyma of Tg6074 mice presenting with neurological disease.

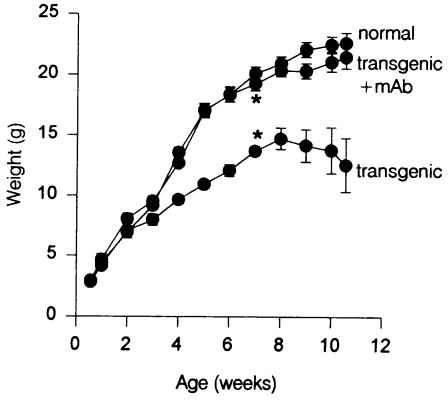

FIG. 3. Neutralization of weight loss in Tg6074 mice by anti-murine TNF-α antibody administration. Untreated (or hamster IgG-treated; not shown) Tg6074 mice develop neurological disease and initially show reduced weight gain followed by weight loss. In contrast, anti-murine TNF-α antibody-treated Tg6074 mice showed no signs of neurological disease and displayed normal weight gain. The results are expressed as the mean ± SEM. *Groups of mice analyzed statistically using Student's t test ($P < 0.002$).

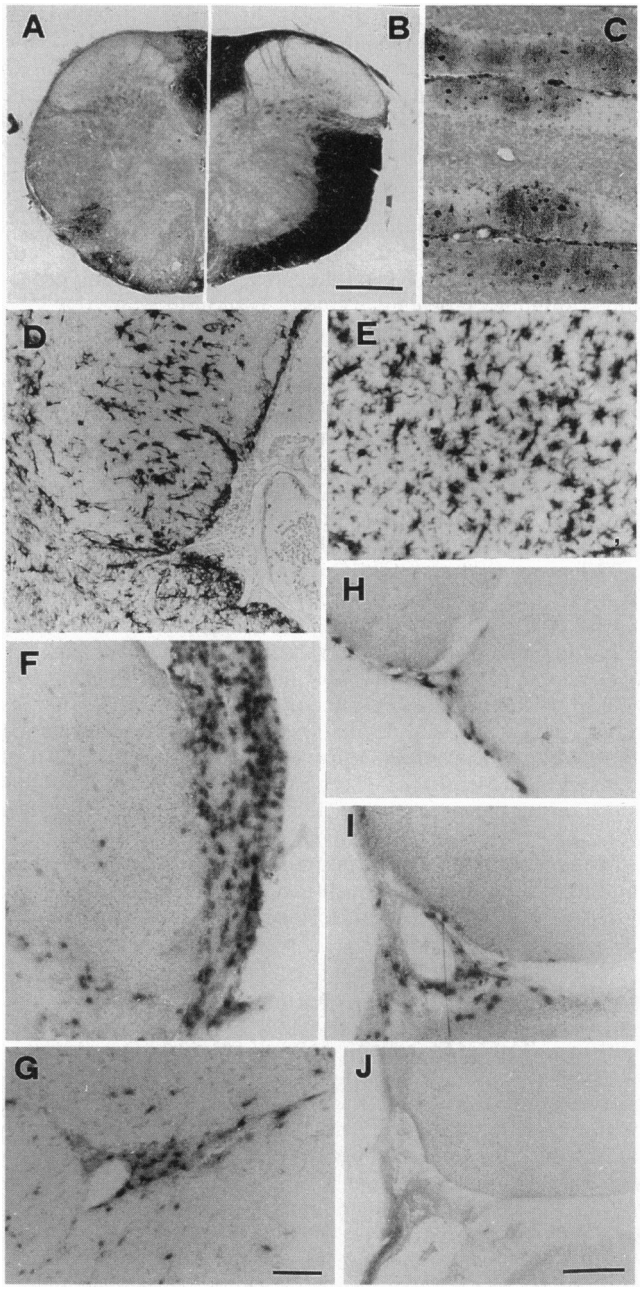

FIG. 4. Histopathological changes in the CNS of Tg6074 mice. (*A*) Cervical spinal cord from an 8-month-old animal (oil red/hematoxylin) showing extensive demyelination. (*B*) Normal control. (*C*) Cerebellum from a 3-month-old animal (oil red/hematoxylin) showing numerous lipid-laden macrophages. GFAP immunostained astrocytes in the thalamus (*D*) and CD18-immunoreactive microglia in the cortex (*E*), demonstrating reactive gliosis. CD4- (*F*) and CD8- (*G*) immunoreactive T cells infiltrating the hypothalamus and anterior thalamus, respectively, of an animal with severe neurological disturbances. CD4- (*H*) and CD8- (*I*) immunoreactive T cells limited to the subarachnoid space in the cerebellum of antibody-treated animals. (*J*) CD4 immunostaining in a normal control brain. (*A* and *B*, bar = 100 μm; *C–E* and *H–J*, bar = 25 μm; *F* and *G*, bar = 15 μm.)

Neutralization of the Tg6074 Phenotype by Administration of Anti-Murine TNF-α Monoclonal Antibodies. The neurological phenotype and weight loss exhibited by Tg6074 mice were not associated with detectable serum levels of murine

TNF-α, as determined by ELISA (sera tested from 14 mice, sensitivity of assay 10 pg/ml; data not shown). However, both the neurological phenotype and weight loss were completely neutralized by the intraperitoneal administration of a neutralizing anti-murine TNF-α antibody (Fig. 3). Moreover, histopathological analysis of CNS tissues from antibody-treated mice revealed no evidence of reactive astrocytosis, demyelination, or T-cell infiltration into the CNS parenchyma, and only low numbers of microglia were observed. Interestingly, significant numbers of T cells were localized within the subarachnoid space at the surface of the brain (Fig. 4 *H* and *I*) and spinal cord of antibody-treated animals when compared to normal controls (Fig. 4*J*).

DISCUSSION

We have generated transgenic mice expressing a murine TNF-α transgene specifically in their CNS, which spontaneously develop chronic inflammatory demyelinating disease with 100% phenotypic penetrance from around 3 to 8 weeks of age. Mice die prematurely (within ≈8 months) after developing progressively severe neurological symptoms ranging from mild tremors and ataxia to severe imbalances and seizures. Other symptoms include loss of the limb flexion reflex, hind limb paresis, and kyphosis. Histopathological features were reactive astrocytosis and microgliosis, infiltration of the meninges and CNS parenchyma with $CD4^+$ and $CD8^+$ T lymphocytes, and focal demyelination. Interestingly, the intraperitoneal administration of a monoclonal antibody to murine TNF-α resulted in neutralization of the phenotype and amelioration of histopathological changes, indicating a direct role for TNF-α in the development of disease.

Expression analysis of Tg6074 mice showed that the murine TNF-α transgene is expressed from approximately P6 and that expression appears to be restricted to a subpopulation of CNS neurons. Previous immunocytochemical studies have suggested that neurons are the principal TNF-α-producing cell type in the normal murine brain (5). However, the CNS-specific expression of the murine TNF-α transgene used in this study is surprising since several other similarly structured human and murine TNF-α transgenes (refs. 21 and 25 and our unpublished observations) never showed such strict expression specificity for the CNS. Moreover, transgene expression is first detectable in the CNS of P6 mice and increases until ≈P15, from when on it remains at high levels. It seems likely, therefore, that this developmentally regulated and CNS-specific expression pattern of the transgene is ectopic and occurs as a result of a position effect exerted on the transgene by potent CNS-specific cis-acting DNA regulatory sequences found in the proximity of the transgene integration site in the mouse genome.

The presence of infiltrating $CD4^+$ and $CD8^+$ T cells was a prominent feature in the CNS of Tg6074 mice from as early as 3 weeks of age. T-cell entry into the CNS parenchyma appears to be strictly regulated by the blood–brain barrier under normal conditions but is markedly increased in inflammatory diseases such as MS (27) and experimental autoimmune encephalomyelitis (EAE) (28). TNF-α overexpression in the CNS may directly influence T-cell trafficking at the blood–brain barrier. *In vitro*, TNF-α induces adhesion molecule expression on vascular endothelial cells and selectively enhances their adhesiveness for T cells (see ref. 4). Antibodies to cell adhesion molecule ICAM-1, for example, can inhibit EAE (29). Our observation that T-cell infiltration into the CNS parenchyma of Tg6074 mice is prevented by intraperitoneal anti-TNF-α antibody treatment indicates that one action of the antibody may be to reduce barrier permeability to T cells through the local neutralization of TNF-α action. Recruitment of T cells within the subarachnoid space, however, is not completely prevented by antibody treatment, suggesting that different barrier mechanisms operate here. Recent evidence has suggested that the subarachnoid space is an important early site for precursor T-cell proliferation and CNS infiltration in EAE (30), and it is possible that a similar pattern of infiltration takes place in Tg6074 mice.

The T-cell infiltrate is believed to be central to disease pathogenesis in inflammatory CNS diseases such as MS (27, 31) and cerebral malaria (12). In MS T-cell infiltration is one of the initial events in disease after breakdown of the blood–brain barrier (27, 31). The prevailing belief is that MS is primarily autoimmune in nature, involving an integrated attack by T cells, B cells, and macrophages on the myelin sheath. However, evidence for this is largely circumstantial and derives mainly from studies of EAE, where disease is transferrable by T cells bearing myelin protein-specific T-cell receptors (see ref. 28). Nevertheless, transgression of the blood–brain barrier is also a key event in EAE. Encephalitogenic T cells administered intrathecally, instead of intravenously, fail to induce disease (32). It is relevant here that disease in Tg6074 mice is neutralized by peripherally applied anti-TNF-α antibody. Although we cannot exclude that antibody crosses a leaky blood–brain barrier to neutralize TNF-α at the site of transgene expression, this seems unlikely during long-term treatment, since repair of barrier function and consequent limited penetrance of antibody would also be expected. It seems more probable that TNF-α-triggered infiltration of the CNS by inflammatory cells is an important initial event in disease pathogenesis in Tg6074 mice. It remains to be determined whether the CNS-infiltrating T cells in Tg6074 mice can also be pathogenic, as in EAE, and, if so, whether they develop restriction to CNS-specific antigens.

Reactive astrocytosis and reactive microgliosis are also predominant histopathological features of Tg6074 mice presenting with neurological symptoms. Reactive gliosis is a characteristic response of the adult CNS to trauma, infection, and inflammation (33, 34) and is seen in EAE (35) and in MS lesions (36). Cytokines are believed to be major effectors in this process (37–40). Injections of TNF-α stimulate the appearance of reactive astrocytes (39) and microglial *in vivo* (41). Moreover, TNF-α induces astrocyte proliferation and activation *in vitro* (42, 43). Therefore, it may be hypothesized that reactive gliosis in the Tg6074 mice is a consequence of deregulated overproduction of TNF-α. Considering evidence that gliosis may have detrimental effects in the CNS (1, 44), a key role of gliosis in the pathogenesis of the neurological phenotype in Tg6074 transgenic mice may also be conceived. In particular, microglia appear to possess key properties for a role in immune-mediated inflammation, among them their ability to express major histocompatibility complex class II molecules and act as antigen-presenting cells (45) and their ability to confer EAE susceptibility to otherwise resistant CNS tissues when activated (46).

The myelin disruption induced in aged Tg6074 mice substantiates previous *in vitro* evidence that has implicated TNF-α as a key effector of immune-mediated demyelination. TNF-α, along with lymphotoxin, is selectively cytotoxic to primary oligodendrocytes (15) and causes myelin damage in murine CNS explants (16, 47). TNF-α-immunoreactive inflammatory cells and astrocytes are present in MS plaques (7), and disease progression in MS has been correlated with high cerebrospinal fluid levels of TNF-α (48). Therefore, demyelination in Tg6074 mice might occur as a direct result of transgene expression or as a bystander effect of local activated astrocytes, macrophages/microglia, and infiltrating T cells. Alternatively, TNF-α may act alone or in series with other proinflammatory cytokines such as interleukin 1 (49) to induce the production of further inflammatory molecules such as nitric oxide, which may, in turn, induce oligodendrocyte death (50, 51) and affect the normal differentiation and growth of neuronal cell precursors (51).

The evidence for a major involvement of TNF-α in the pathogenesis of inflammatory demyelinating diseases such as MS and EAE is now strong, and many therapeutical approaches target the action of this cytokine. EAE can be exacerbated by exogenous TNF-α (52) and neutralized by several anti-TNF-α and anti-TNF-α/lymphotoxin strategies (8, 53–55). EAE is also inhibited by interleukin 4 treatment, which induces a switch from the TH1 (TNF-α-producing) to the immune-suppressive TH2 helper cell phenotype (56) and the antidepressant rolipram, which inhibits T-cell activation and TNF-α secretion (57). We demonstrate here that the overexpression of TNF-α in the CNS of transgenic mice can trigger the development of a chronic inflammatory demyelinating disease. The observed reactive astrocytosis, reactive microgliosis, aberrant T-cell entry into the CNS, and myelin disruption are all characteristic features of human inflammatory and/or demyelinating CNS diseases. The TNF-α transgenic mice described in this study may represent an invaluable model system to aid further studies and therapeutical management of such diseases in humans.

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