Impairment of TNF-Receptor-1 Signaling but not Fas Signaling Diminishes T-Cell Apoptosis in Myelin Oligodendrocyte Glycoprotein Peptide-Induced Chronic Demyelinating Autoimmune Encephalomyelitis in Mice

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T-cell apoptosis in inflammatory demyelinating lesions of chronic myelin oligodendrocyte glycoprotein peptide₃₅₋₅₅ induced autoimmune encephalomyelitis was studied in several different gene knockout mice as well as their wild-type counterparts. The gene deletions included tumor necrosis factor (TNF) α , lymphotoxin, TNF receptor 1 or 2, Fas-L, inducible nitric oxide synthase, perforin, and interleukin1 β -converting enzyme. Impairment of the TNF receptor 1 pathway led to a 50% reduction of T-cell apoptosis in the central nervous system lesions, whereas the other genetic deletions showed no significant effect. Our study thus identified the TNF receptor 1 signaling pathway as one mechanism responsible for the removal of T lymphocytes from inflammatory demyelinating lesions of the central nervous system. (Am J Pathol 1999, 154:1417-1422)

Apoptosis of T lymphocytes in the central nervous system (CNS) appears to be a key element in the down-regulation of autoimmune brain inflammation.¹ Both antigen-specific and antigen-independent mechanisms² have been suggested to trigger T-cell apoptosis. Possible immunological mediators for the induction of T-cell apoptosis are activation of death signaling through FAS ligand (FasL) and Fas³ or the TNF lymphotoxin-TNF receptor-1 (TNFR1) pathway,⁴ cytotoxic T-cell interactions⁵ involving target cell killing by granzymes and perforin, or cytotoxicity mediated by nitric oxide radicals.⁶ Alternatively, deprivation of costimulatory factors and growth factors may lead to apoptosis of activated T cells invading the CNS. To define the role of these different mechanisms in CNS inflammation, we studied experimental autoimmune en-

cephalomyelitis (EAE) in mice deficient in different immune effector molecules.

Our data suggest the involvement of two pathways. A deletion of the TNFR1 signaling pathway was found to be associated with a 50% reduction in the rate of T-cell apoptosis in the lesions. In contrast, inactivation of the Fas and perforin pathway had no such effect. Besides TNFR1-mediated apoptosis, the CNS tissue disposes of an additional apoptotic induction mechanism for T cells independent of known immunological pathways.

Materials and Methods

Mice

Besides the respective wild-type animals, mice with genetic deletion of TNF and lymphotoxin, perforin, iNOS, interleukin-1 β -converting enzyme, FasL, TNFR1, TNFR2, and TNFR1/2, as well as animals with transgenic expression of soluble TNFR1, were used in this study (Table 1). The animals were bred and housed in the animal facilities of the Institute of Laboratory Animal Science of the University of Zurich and of Biological Research Laboratories, Ltd. (Füllinsdorf, Switzerland).

Active Induction of EAE

Myelin oligodendrocyte glycoprotein (MOG) peptide₃₅₋₅₅, corresponding to amino acid residues of rat and mouse MOG sequence,⁷ was synthesized by Chiron Technologies (Clayton, Victoria, Australia). Mice were injected s.c. at the left side flank with 0.3 ml of emulsion composed of 0.3 mg MOG₃₅₋₅₅ dissolved in 0.15 ml phosphate-buffered saline (PBS) (pH 7.4) and 0.75 mg of *Mycobacterium tuberculosis* (H37Ra, Difco, Detroit, MI) in

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Table 1. Knockout Mouse Species

Knockout animals (abbreviation)	Wild-type counterparts	Gene deletion (knockout)	References (no.)	EAE studies
gld-mice	C57BL/6	gene mutation: express FasL in an inactive form	Braun et al (22)	Malipiero et al (10)
sTNFR1 TG (sTR1)	C57BL/6	soluble TNF-receptor-1 transgenic	Garcia et al (27)	
perforin ^{-/-}	C57BL/6	perforin	Braun et al (22)	Malipiero et al (10)
ICE ^{-/-}	129SvxC57BL/6	interleukin 1 β -converting enzyme	Kuida et al (23)	
TNFR1 ^{-/-} (TR1)	129SvxC57BL/6	TNF receptor-1	Rothe et al (24)	Eugster et al (9)
TNFR2 ^{-/-} (TR2)	129SvxC57BL/6	TNF receptor-2	Erickson et al (25)	Eugester et al (9)
TNFR1/2 ^{-/-} (TR12)	129SvxC57BL/6	TNF receptor-1 and -2 double knockout (generated by intercrossing TR1 and TR2)	Bluethmann (unpublished)	Eugster et al (9)
TNF/L Ta ^{-/-} (TNLT)	129SvxC57BL/6	TNF and lymphotoxin double knockout	Eugster et al (26)	Eugster et al (9)
iNOS ^{-/-}	129SvxC57BL/6	inducible nitric oxide synthase	MacMicking et al (28)	Sahrbacher et al (11)

0.15 ml incomplete Freund's adjuvant (Difco). On the day of immunization and 2 days later, 500 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) were injected i.p. in 0.4 ml pertussis diluent (0.15 M Tris, 0.5 mol/L NaCl and 0.017% (v/v) Triton X-100 in distilled water). Immunization was repeated 1 week later with 0.3 mg MOG on the right flank of the mice without pertussis toxin.

Clinical Evaluation of EAE

After the booster immunization the mice were monitored and weighed daily. Clinical manifestations of EAE recorded on a scale of 0 to 5, with intermediate clinical findings. Scores designated the following: 0, no detectable signs of EAE; 0.5, distal limp tail; 1, complete limp tail; 1.5, limp tail and hind limb weakness (unsteady gait and poor grip of hind legs); 2, unilateral partial hind limb paralysis; 2.5, bilateral partial hind limb paralysis; 3, complete hind limb paralysis; 3.5, complete hind limb paralysis and unilateral forelimb paralysis; 4, total paralysis of hind and forelimbs (mice at grade 4 >1 day were euthanized); 5, death.

Mice showing signs of paralysis were given easy access to food and water and, to prevent dehydration, s.c. injections of sodium chloride (0.9%) were administered if required. The animal experiments were approved by the local authorities and performed according to the institutional guidelines.

Histological Analysis

Animals were anesthetized, bled, and perfused with 25 ml Ringer solution (Braun Medical AG, Emmenbrücke, Switzerland) and 10 ml 4% paraformaldehyde in buffered PBS. Brain and spinal cord were dissected out and fixed overnight in 4% paraformaldehyde in buffered PBS at 4°C before embedding in paraffin. Paraffin-embedded tissue sections (3–5 μ m) were stained with hematoxylin and eosin and Luxol fast blue myelin stain. DNA fragmentation in paraffin sections was visualized by *in situ* tailing.⁸

Immunocytochemistry was performed on 3- μ m-thick paraffin sections using a biotin/avidin/peroxidase tech-

nique and the following primary antibodies: anti-human/mouse CD3 cross-reacting with mouse CD3-(Serotec, Oxford, UK) and anti-mouse CD45 Ab (Pharmingen, San Diego, CA).

Methods of Quantitative Evaluation

Quantitative studies were performed on an average of 12 cross-sections of different spinal cord levels. The average cross-section area of the spinal cord (mm²) was determined at a magnification of 2.5 by overlaying the sections with a stereological grid and counting the intersections lying within.

The inflammatory index was defined as the number of perivascular inflammatory cuffs per millimeter squared in the spinal cord.

The size of demyelinated lesions was determined by counting the intersections of a stereological grid overlying the demyelinated area in comparison to the normal spinal cord tissue, in sections stained with Luxol fast blue myelin stain.

To evaluate T-cell apoptosis, we first determined the number of T cells visualized by immunocytochemistry for CD3. To distinguish between apoptotic and nonapoptotic T cells, we applied morphological criteria defining apoptotic T cells through cell shrinkage, chromatin condensation, and nuclear fragmentation. Four hundred cells per animal were analyzed and categorized in normal CD3+ T cells, apoptotic CD3+ cells, and unlabeled apoptotic cells. The final values are given in percentages of total T cells. DNA fragmentation in apoptotic T cells was visualized by double labeling with *in situ* tailing⁸ and immunocytochemistry for CD3.

To define lesional activity, the presence of macrophages with intracytoplasmic myelin degradation products was evaluated. The following score was used: 0, no activity (demyelination, no macrophages with myelin degradation products); 1, moderate activity (infiltration of myelin degradation product-containing macrophages in perivenous or subpial regions or at plaque edges); 2, severe activity (confluent demyelinated plaques, high density of macrophages with myelin degradation products throughout the lesions).

Table 2. Quantitative Analysis of Inflammation and Demyelination

Animals	Inflammation			Demyelination in chronic stages		
	No. of animals	Mean value	Standard error	No. of animals	Mean value	Standard error
wt B6	12	2.04	±0.580	6	4.75	±1.21
gld	13	0.55*	±0.313	13	1.638*	±0.59
per	9	3.237	±0.546	8	7.25	±2.00
sTR1	13	1.704	±0.634	8	2.65	±1.87
wt B6x129	13	0.96	±0.290	9	11.62	±3.07
iNOS	5	3.32*	±0.159	n.a.	n.a.	n.a.
ICE	21	0.99	±0.256	18	5.14	±1.37
TNLT	7	1.986*	±0.458	7	1.071*	±0.241
TR1	7	1.457	±0.384	7	1.114*	±0.338
TR2	7	0.771	±0.286	4	5.45	±1.650
TR12	6	0.5	±0.210	6	1.183*	±0.490

n.a., not available; all iNOS^{-/-} animals died in the acute stage.

*Significance level compared with the respective wild-type mouse, $P < 0.05$.

Results

T-Cell Apoptosis in Chronic EAE in Wild-Type Mice

Immunization with MOG₃₅₋₅₅ in Complete Freund's Adjuvant induced a chronic demyelinating form of EAE in all wild-type animals, irrespective of their genetic background (C57BL/6 *versus* C57BL/6xSv129 F1). The acute phase of the disease (days 10–30) was dominated by perivenous inflammation accompanied by mild perivenous demyelination. At later stages of the disease, demyelination became prominent, leading to confluent demyelinated plaques (Table 2). As described recently, inflammation was increased in TNF/LTa^{-/-} and

TNFR1^{-/-} compared to the respective wild-type C57BL/6x129Sv mice.⁹ This contrasts with low demyelination in TNF/LTa^{-/-} and TNFR1^{-/-} compared to TNFR2^{-/-} and the respective wild-type mice. In ICE^{-/-} mice, the inflammatory response in the CNS was not different from the controls. Perforin^{-/-} and iNOS^{-/-} mice developed a more severe form of EAE than their wild-type counterparts. As reported, the Fas/FasL mutations (gld- and lpr-mice) were associated with an acute transient form of disease without chronic inflammation in the CNS.^{10,11} Apoptosis of T lymphocytes, determined by either morphological criteria (Figure 1, a and d) or visualization of DNA fragmentation (Figure 1, b and c), was found throughout all stages of the disease. The rate of

Figure 1. a: Apoptosis in CD3⁺ T-cells in MOG EAE. The different stages in apoptotic CD3⁺ T cells in an inflammatory infiltrate are defined through morphological criteria (arrowheads): condensation and margination of chromatin and nuclear fragmentation. Immunocytochemistry for CD3 (brown), nuclear stain with hematoxylin (blue) (×1000). b, c: Double labeling for apoptotic T cells (arrowhead) with CD3-antibody and *in situ* tailing to visualize DNA fragmentation (black-stained nucleus) in a larger magnification (×1250). d: Apoptotic CD3⁺ T cells with nuclear fragmentation in a higher magnification (×1250).

Figure 2. Percentage of T-cell apoptosis in various models of EAE: significant difference (*) in the rate of T-cell apoptosis for the TNLT^{-/-}, the TR1^{-/-} and the TR12^{-/-} model compared to the respective wild-type mice. wt B6, wild-type B6; wtX, wild-type B6x129.

T-cell apoptosis ranged between 5% and 20% of the local T lymphocyte population. No statistically significant differences were found in the percentage of apoptotic T cells between C57BL/6 and C57BL/6x129Sv animals (Figure 2). Similarly, no significant changes in the rate of apoptosis were found in relation to the stage of the disease (days after sensitization) nor to the overall severity of the inflammatory process.

Blockade of the TNFR1 Signaling Pathway Reduces the Rate of T-Cell Apoptosis in EAE

When compared with wild-type animals of comparable genetic background, all experimental groups with an impaired TNFR1 signaling pathway revealed a significant reduction of T-cell apoptosis. Thus, the percentage of apoptotic T cells was reduced by approximately 50% in animals with a deletion either of TNFR1 or of both TNFR1 and TNFR2. Likewise, double knockout animals for both the TNF α and LT α genes showed a 50% reduction of apoptosis. The lack of a functional TNFR2, as well as transgenic expression of soluble TNFR1, also resulted in a decrease of apoptotic T cells. The effect, however, was not statistically significant (Figure 2).

T-Cell Apoptosis in EAE Lesions Is not Influenced by FasL, Perforin, iNOS, or Interleukin1 β -Converting Enzyme

In contrast to inactivation of the TNFR1-mediated pathway, mutation of the FasL, perforin, iNOS, and interleukin1 β -converting enzyme genes did not alter the rate of T-cell apoptosis in the lesions, despite significant differences in the global extent of inflammation and demyelination. This was true when comparing the mutant or transgenic mice with either the pooled control animals or the wild-type animals that displayed a genetic background directly comparable to the mutant animals (Figure 2).

Figure 3. a: Highly significant correlation of T-cell apoptosis to the lesional activity in wild-type animals. **b:** No significant correlation of T-cell apoptosis to the lesional activity in knockout mice with an impairment of the TNFR1 signaling pathway.

Extent of T-Cell Apoptosis Correlates with Demyelinating Activity in Wild-Type Animals but not in Animals with an Impaired TNFR1 Signaling Pathway

Because in a recent study (A. Weishaupt, R. Gold, T. Hartung, S. Gaupp, A. Wendel, D. A. Hafler, and K. V. Toyka, unpublished manuscript) the TNF-R1 signaling pathway was found to be required for the induction of T-cell apoptosis by soluble peptide antigen, we investigated whether antigen liberation in the course of active demyelination in EAE may influence the rate of T-cell apoptosis in the CNS. In wild-type animals, we found a significant positive correlation between the degree of demyelinating activity and the percentage of apoptotic T cells in the lesions. This correlation was completely abolished in animals with impaired TNFR1 signaling pathway (Figure 3).

Discussion

Recent evidence supports the concept that immune privilege in certain organs, such as the brain and eye, is at least partly achieved by the effective elimination of incoming T lymphocytes through apoptosis.¹ In both the brain and the eye, high rates of T-cell apoptosis are invariably present in inflammatory conditions.^{12–14} Destruction by programmed cell death involves not only the target antigen-specific T cell population, but also the secondary recruited T cells with specificities for antigen, not present in the CNS.² In the eye, elimination of T cells may depend at least in part on intact Fas-FasL signaling. Fas-expressing T cells come in intimate contact with local FasL-expressing cells, and deletion of the Fas-signaling pathway significantly reduces the rate of T-cell apoptosis in this organ.^{12,15} In the CNS, FasL is highly expressed on CD4⁺ T cells as well as on local microglia cells under inflammatory conditions.¹⁶ The latter cells form a regular network of cell processes throughout the CNS tissue¹⁷ and contact is therefore likely between them and infiltrating T cells. However, as shown in our present study, deletion of FasL in gld-mice has no effect on the rate of T-cell apoptosis in the CNS.

Similarly, no decrease of T-cell apoptosis has been found in perforin-deficient animals. Thus, despite a possible interaction of autoimmune T cells with regulatory T cells, as described *in vitro*,⁵ neither FasL nor perforin plays a major role in the elimination of inflammatory T cells from the CNS. It is of note that T-cell apoptosis was impaired in neither ICE^{−/−} nor iNOS^{−/−} knockout mice. Reactive oxygen and nitrogen species generated by the activity of iNOS in macrophages can contribute to macrophage-mediated apoptosis of T cells⁶ and target cells. However, their role in T-cell apoptosis induction in the CNS appears to be minor.

Impairment of the TNFR1 signaling pathway, although not blocking T-cell apoptosis in the CNS completely, resulted in a significant 50% reduction of its rate. Thus, our data suggest that the activation of the TNFR1-associated death domain is instrumental for the elimination of a subgroup of T cells within the lesions. Although less obvious, some reduction of T-cell apoptosis was also found in animals with deleted TNFR2. Although TNFR2 does not contain an intracellular death domain itself, it may augment TNFR1-induced apoptosis by interfering with intracellular signaling pathways.^{18,19}

In a recent study, Weishaupt et al²⁰ describe treatment of EAE animals with a high dose of soluble antigen, resulting in a dramatic increase of T-cell apoptosis in their inflammatory CNS lesions. Interestingly, this increase in the T-cell apoptosis rate was completely abolished by blocking TNFR1 (A. Weishaupt, R. Gold, T. Hartung, S. Gaupp, A. Wendel, D. A. Hafler, K. V. Toyka, unpublished manuscript). In contrast, the basic rate of T-cell apoptosis in the lesions of animals not treated with soluble antigen was unaffected.

These data, in conjunction with our present results, suggest that in demyelinating forms of chronic EAE, two independent pathways of T-cell destruction occur in parallel, one of which is TNFR1-dependent. The mechanism

of the other, TNFR1-independent pathway is not yet clear. Absence of costimulatory molecules and lack of growth factors may contribute to this form of T-cell apoptosis. Alternatively, expression of the surface molecule galectine by glia cells may lead to apoptosis.¹ The latter mechanism apparently dominates in acute monophasic EAE following passive transfer of T lymphocytes, a disease which is purely inflammatory² and does not lead to significant demyelination or tissue destruction.

In demyelinating conditions, however, where target tissue is destroyed and the respective antigens are released into the brain extracellular space,²¹ another mechanism appears to operate, eliminating the antigen-specific T-cell population and depending on TNFR1 signaling. Interestingly, a significant correlation was found between the rates of T-cell apoptosis and demyelinating activity in wild-type mice that was absent in animals with impaired TNFR1 signaling.

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