

Astrocyte-induced T cell elimination is CD95 ligand dependent

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Abstract

The brain has an intrinsic capacity to remove infiltrating T cells by inducing apoptosis. However, the pathways and cellular components driving this process are still under debate. Astrocytes seem to play an important role because they colocalize with apoptotic lymphocytes in vivo and induce apoptosis of transformed T cells in vitro. Since we previously demonstrated the expression of the death ligand CD95L (APO-1L/FasL) on astrocytes in the brain, we wanted to know whether nontransformed astrocytes induce cell death in nontransformed T cells, reflecting the in vivo situation and, if so, whether CD95/CD95 ligand interaction is important. T cell apoptosis measured by Annexin V binding and DNA fragmentation was significantly lower using CD95 ligand-deficient (*gld*) astrocytes compared to nondeficient controls. Moreover, neutralizing anti-CD95 ligand antibody reduced astrocyte-induced T cell apoptosis. Thus, adult astrocytes are capable of inducing the apoptotic death of T cells by involving the CD95/CD95 ligand pathway without undergoing cell death in vitro. Since astrocytic end-feet contribute to the formation of the blood–brain barrier, this depletion mechanism may play an important role as the first line of defense in the brain.

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1. Introduction

Inflammation in the brain is terminated by the induction of T cell apoptosis (Tabi et al., 1994; Schmied et al., 1993). While antigen recognition is not necessary for the elimination of infiltrating T cells (Bauer et al., 1998), there is evidence that apoptosis of these T cells depends on the expression of the death ligand CD95L (CD95L, APO-1L, FasL) on brain cells (Waldner et al., 1997; Sabelko et al., 1997; White et al., 1998; Suvannavejh et al., 2000; Sabelko-Downes et al., 1999; Wildbaum et al., 2000). Yet, the exact cellular components involved in the induction of T cell apoptosis within the brain are not known. We and others

have previously described the expression of the CD95 ligand on astrocytes, its upregulation after brain lesion and the capacity of astrocytes to kill CD95-expressing malignant T cells known to be extremely sensitive to apoptosis (Gold et al., 1996; Saas et al., 1997; Choi et al., 1999; Bechmann et al., 1999, 2000). Indeed, CD95 ligand-expressing astrocytes are closely situated to apoptotic T cells during experimental allergic encephalomyelitis (EAE), an animal model of multiple sclerosis (Kohji et al., 1998; Kohji and Matsumoto, 2000), again suggesting an astrocytic capacity to kill invading T cells. To clarify whether these astrocytes are capable of inducing apoptosis in nontransformed T cells and whether this mechanism is CD95 ligand dependent, we compared the capacity of CD95 ligand-deficient (*gld*) and nondeficient astrocytes to induce apoptosis in activated CD95-deficient (*lpr*) and -nondeficient T cells. The rate of apoptosis was measured by Annexin V binding and DNA fragmentation (Nicoletti et al., 1991). Additionally, a neutralizing anti-CD95 ligand antibody was used to specifically block CD95 ligand-induced apoptosis.

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2. Materials and methods

2.1. Animals

C57 BL/6J, B6SMN.C3H CD95L (*gld*) and B6.MRL CD95 (*lpr*) mice were purchased from the Jackson Laboratory (USA). The animals were housed under standard conditions with free access to food and water. Care was taken to minimize any pain and discomfort for the animals.

2.2. Preparation of astrocytes

Primary cultures of mixed cortical glia were prepared from neonatal C57BL/6J and B6SMN.C3H CD95L (*gld*) mice using the method described by Giulian and Baker (1986) with minor modifications. In brief, after removal of the meninges, cerebral cortices were mechanically dissociated with a glass pipette in HBSS without Ca^{2+} and Mg^{2+} (HBSS⁻; Gibco BRL, Life Technologies, Germany), containing a mixture of trypsin (Boehringer, Germany) and DNase (Worthington, USA). After further titration, cells harvested from two cortices were plated into six-well plates (Falcon, Germany) containing Dulbecco's modified eagle medium (DMEM; Gibco) with 4.5 g/l glucose and without sodium pyruvate supplemented with 10% fetal calf serum (FCS; Gibco), 1% L-glutamine (Gibco), 100 U/ml penicillin (Sigma, Germany) and 0.1 mg/ml streptomycin (Sigma). After primary incubation for 3 days (37 °C; 5% CO_2), the cells were washed with HBSS and maintained in fresh DMEM as described above. Cultures were fed twice weekly and shaken to remove adherent microglial cells from the astrocytic monolayer. Upon reaching confluence, the cells were cocultured with T lymphocytes as described below.

2.3. Preparation of T cells

Spleens and lymph nodes from C57BL/6J and B6.MRL CD95 (*lpr*) mice were homogenized and washed. Erythrocytes were lysed in 0.83% NH_4Cl . CD4 T cells were isolated in a Midi MACS column (Miltenyi Biotec, Germany) after binding to superparamagnetic microparticles conjugated with monoclonal antibodies (anti-CD4 MACS beads; Miltenyi Biotec). The cells were activated overnight by incubation with phorbolmyristatacetate (PMA; 5 ng/ml; Sigma) and ionomycin (1 $\mu\text{g}/\text{mg}$; Sigma) in RPMI-1640 (Gibco) supplemented with 10% fetal calf serum (FCS; Gibco), 1% L-glutamine (Gibco), 100 U/ml penicillin (Sigma) and 0.1 mg/ml streptomycin (Sigma) for 48 h (37 °C; 5% CO_2).

2.4. Cocultures of astrocytes and T lymphocytes

Initially, between 10^5 and 10^6 T lymphocytes from C57BL/6J, B6SMN.C3H CD95L (*gld*) or B6.MRL CD95 (*lpr*) were seeded in six-well plates either alone as a

control for background apoptosis due to stimulation, or on astrocytic monolayers for 4, 12 and 24 h. T cells were then removed and apoptosis was determined by Annexin V binding and DNA fragmentation. Since the astrocyte-induced T cell apoptosis had its maximum at 12 h, all experiments were then performed with 10^5 T cells and an incubation time of 12 h. To block the CD95 pathway, an antagonistic CD95 ligand antibody (MFL3; Pharmingen, Germany) was applied at a concentration of 5 $\mu\text{g}/\text{ml}$ and its effects were compared to an IgG κ 1 isotype control (Pharmingen) used at the same concentration.

2.5. Annexin V binding

Phosphatidylserine exposure of T cells was evaluated by Annexin V-FITC staining. Briefly, 2×10^5 cells per sample were washed with phosphate buffer solution (PBS) and incubated in a solution of 0.5 $\mu\text{g}/\text{ml}$ FITC-labeled Annexin V (Becton Dickinson, Germany) in binding buffer (140 mM NaCl, 2.5 mM CaCl_2 , 10 mM HEPES/NaOH; pH 7.4) at +4 °C for 15 min. The percentage of Annexin V binding cells was analyzed by flow cytometry. Samples were measured using a FACScan flow cytometer and analyzed using the Lysis II software (Becton Dickinson, USA).

2.6. DNA fragmentation assay

Quantitative analysis of DNA fragmentation was performed as described elsewhere (Nicoletti et al., 1991). Briefly, a hypotonic fluorochrome solution [propidium iodide (PI) 50 $\mu\text{g}/\text{ml}$ in 0.1% sodium citrate and 0.1% Triton X-100] was added to 10^5 cells treated as indicated and followed by incubation for 4 h at 4 °C. The magnitude of the hypodiploid DNA peak was determined as the percent of total events by flow cytometry.

2.7. Immunocytochemistry

To test the viability of astrocytes after cocultivation with T cells, T cells were removed and the astrocytic monolayers were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature and stored in PBS at 4 °C. For fluorescence staining, cells were incubated in a solution of 0.5% Triton X, 0.2% normal horse serum and guinea pig anti-GFAP as primary antibody (Advanced ImmunoChemical, USA) in PBS at a concentration of 1:400 overnight at 4 °C. Cells were then washed in PBS for 30 min and incubated in a solution of 0.5% Triton X, 0.2% normal horse serum and anti-guinea pig fluorescein coupled secondary antibody (Sigma) at a concentration of 1:250 in PBS at room temperature for 2 h. Cells were again washed in PBS for 30 min. To determine nuclear fragmentation propidium iodide, staining was performed using PI (Sigma) at a concentration of 2 mg/ml. Double fluorescence microscopy was performed on an Olympus BX-50 microscope. Narrow-band filters were used to guarantee the selectivity of

fluorescence signals. Pictures were taken using an Olympus digital camera.

2.8. Statistical analysis

Levels of Annexin V binding and DNA fragmentation were compared within the subgroups by the nonparametric Mann–Whitney *U*-test.

3. Results

3.1. Apoptotic elimination of T cells induced by astrocytes

To investigate whether nontransformed astrocytes are capable of inducing apoptosis in nontransformed T cells,

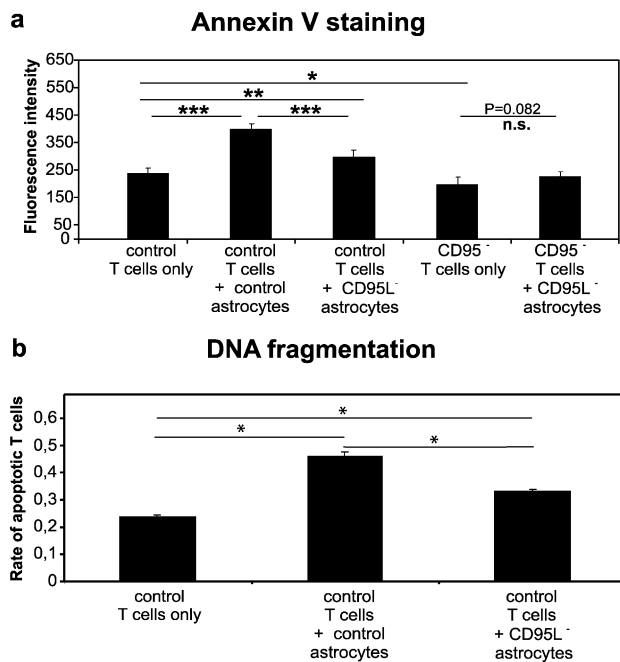


Fig. 1. Apoptotic elimination of T cells induced by astrocytes. Data were analyzed using the Mann–Whitney *U*-test. T cells (10^5) were monocultured or cocultured on astrocyte monolayers for 12 h. Hereafter, CD4⁺ T cells were harvested from the adherent astrocytic monolayer. (a) Annexin V binding as an early event of apoptosis. Compared to monocultured T cells, T cells in coculture with astrocytes show increased mean Annexin V fluorescence ($***p < 0.0004$). This is also held true for coculturing with CD95L⁻ astrocytes ($**p < 0.001$). However, apoptosis induced by CD95L⁺ astrocytes is significantly higher than apoptosis induced by CD95L⁻ astrocytes ($***p < 0.0001$). Astrocyte-induced T cell apoptosis is below statistical significance in cocultures of CD95⁻ T cells with CD95L⁻ astrocytes ($p < 0.082$; n.s.: not significant). As anticipated, such monocultured CD95⁻ T cells show less Annexin V labeling than control T cells ($*p < 0.034$). (b) DNA fragmentation as a late event of apoptosis. The DNA fragmentation assay showed similar effects of CD95L⁺ expressed on astrocytes, as seen in the Annexin V experiments. Significantly increased T cell apoptosis can be observed after coculturing with CD95L⁺ astrocytes ($*p < 0.0495$). This increase remains significant after coculturing with CD95L⁻ astrocytes ($*p < 0.0495$). However, CD95L⁻ astrocytes are significantly less capable of killing control T cells than nondeficient astrocytes ($*p < 0.0495$).

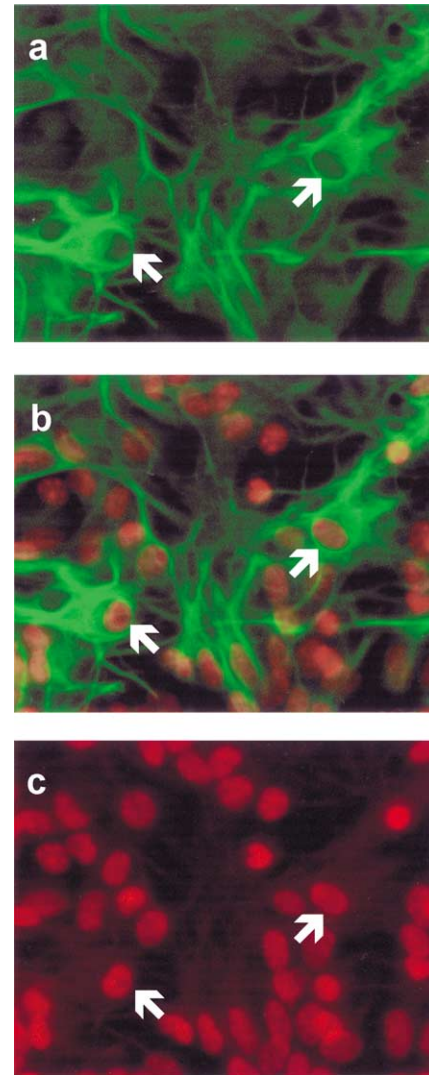


Fig. 2. Viability of astrocytes after coculture with T cells. After removal of T cells, astrocytes were labeled with anti-GFAP (a) and their nuclei were visualized using PI (c). This allowed to search for fragmented nuclei within GFAP-positive astrocytes (b). The arrows point to clearly intact nuclei and the surrounding somata, respectively. No evidence for astrocytic apoptosis was detected.

we measured Annexin V binding, an early event in the apoptosis cascade, and DNA fragmentation, a hallmark of apoptosis in the late phase of programmed cell death. In all experiments, the rate of apoptosis in PMA/ionomycin-activated CD4⁺ T cell monocultures was examined in order to differentiate between the intrinsic fratricide and suicide of T cells, and astrocyte-induced apoptosis. Comparison of monocultured T cells and T cells cocultured with astrocytes yielded a highly significant increase in the apoptosis of cocultured T cells as shown with both Annexin V binding ($p = 0.0004$; Fig. 1a) and DNA fragmentation ($p = 0.0495$; Fig. 1b). Thus, mouse astrocytes induce apoptosis of activated CD4⁺ T cells. In accordance to earlier reports (Becher et al., 1998; Saas et al., 1999a,b; Lee et al., 2000), the astrocytes exhibited no signs of apoptotic cell death after

coincubation with T cells as demonstrated by colocalization of intact nuclei within GFAP positive cells (Fig. 2).

3.2. CD95/CD95 ligand interactions are involved in astrocyte-induced T cell apoptosis

Next, we investigated our hypothesis that CD95/CD95 ligand interaction might play an important role in the observed T cell apoptosis induced by astrocytes. To determine the functional role of astrocytic CD95 ligand, T cell apoptosis induced by CD95 ligand-expressing astrocytes was compared to T cell apoptosis induced by CD95 ligand-deficient *gld* astrocytes. There was a significant difference for both Annexin V binding ($p=0.0001$; Fig. 1a) and DNA fragmentation ($p=0.0495$; Fig. 1b). Thus, *gld* astrocytes are significantly less capable of lysing activated T cells than the nondeficient controls, indicating the importance of the CD95/CD95 ligand system for astrocyte-induced T cell apoptosis.

T cells in monocultures showed significantly less Annexin V fluorescence ($p=0.001$) and less DNA fragmentation ($p=0.0495$) compared to T cells cocultured with CD95 ligand-deficient astrocytes (Fig. 1a and b). This increased apoptosis of T cells induced by the deficient astrocytes may reflect CD95 independent apoptosis-inducing mechanisms or remaining CD95 ligand activity, which is abrogated when CD95 ligand-deficient astrocytes are cocultured with CD95-deficient T cells: as anticipated, such CD95-deficient T cells showed less Annexin V binding ($p=0.034$; Fig. 1a) than control T cells in monocultures; CD95-deficient T cells cocultured with CD95 ligand-deficient astrocytes no longer revealed significantly increased apoptosis when compared to monocultured CD95-deficient T cells ($p=0.082$; Fig. 1a). Thus, enhanced apoptosis of T cells in cocultures with astrocytes crucially depends on an intact CD95/CD95 ligand pathway.

3.3. Astrocyte-induced T cell death critically depends on the CD95 system

As further proof of the importance of CD95/CD95 ligand interactions for the observed astrocyte-induced T cell apoptosis, CD95 ligand was blocked using a neutralizing anti-CD95 ligand antibody. Apoptosis of control T cells exposed to control astrocytes was measured via DNA fragmentation both in the presence and absence of the antagonistic antibody and compared to the rate of apoptosis induced by CD95L-deficient astrocytes. After 12 h, DNA fragmentation in the T cells was measured as in previous experiments. The treatment with the antagonistic antibody (but not with an IgG κ 1 isotype control, not shown), significantly reduced astrocyte-induced apoptosis ($p=0.0063$, Fig. 3) to a level that did not significantly differ from that induced by CD95L-deficient astrocytes ($p=0.9$; Fig. 3).

4. Discussion

For the first time, we clarify the astrocyte-mediated molecular mechanisms of T cell elimination as part of the brain's immune privilege by using cocultures of primary murine astrocytes with activated nontransformed T cells derived from *lpr*, *gld* and control mice. Apoptotic death in T cells induced by astrocytes is reduced when both the effector and target cells lack CD95 ligand or CD95 receptor, respectively (Fig. 1a and b), indicating the importance of the CD95 system and the expression of CD95L on glial cells in defending the brain from invading T cells. As described by several groups, astrocytes constitutively express CD95 ligand (Choi et al., 1999; Bechmann et al., 1999; Lee et al., 2000) and upregulate its expression upon brain lesion, while themselves resisting apoptosis, unless high levels of proinflammatory signals are available (Becher et al., 1998; Saas et al., 1999a,b; Lee et al., 2000; Bechmann et al., 2000). Accordingly, in chronic inflammatory brain lesions of patients suffering from multiple sclerosis, CD95 ligand was predominantly found on glial cells. Although believed to include astrocytes (Dowling et al., 1996), contradictory data exist regarding the identification of these glial cells (D'Souza et al., 1996). In fact, the apoptosis-inducing properties of adult astrocytes can be blocked by an antagonistic antibody of the CD95 ligand, confirming the role of the CD95/CD95 ligand pathway in this cell/cell interaction. However, our finding that CD95 ligand-deficient astrocytes also induce T cell apoptosis, yet at significantly lower levels than normal astrocytes (Fig. 1a and b), can be explained by alternate pathways, which may involve other death receptor/death ligand systems. Since astrocyte-induced apoptosis was no longer significant in cocultures of CD95-deficient astrocytes with CD95-deficient T cells, it is possible that such alternate pathways enhance T cell susceptibility to CD95 ligand and, thus, enhance suicide and fratricide of the T cells.

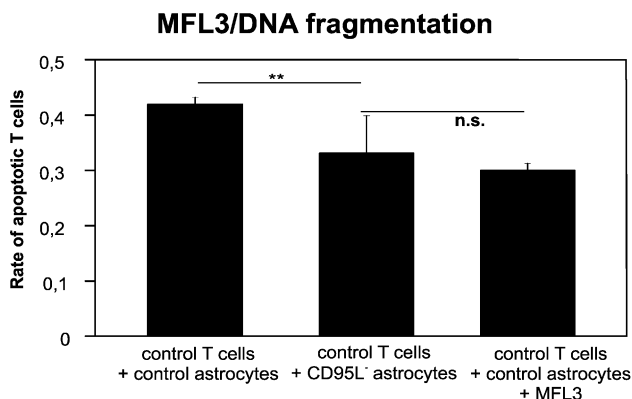


Fig. 3. Blockade of astrocyte-induced T cell apoptosis by a neutralizing anti-CD95L antibody (MFL3). T cell apoptosis (measured by DNA fragmentation) differs significantly in nondeficient and CD95L⁻ astrocytes ($*p<0.0063$). This CD95L⁺ astrocyte-induced T cell apoptosis is completely diminished in cocultures of nondeficient astrocytes with T cells by application of neutralizing anti-CD95L antibody MFL3 ($p<0.9$; n.s.: not significant). Thus, astrocytes kill more T cells in the presence of functional CD95L.

It is noteworthy that the actual rate of T cell apoptosis might be higher than detected due to phagocytosis of dead lymphocytes by astrocytes. Nevertheless, we provide clear evidence that the expression of CD95 ligand on astrocytes previously described by us and others (Choi et al., 1999; Bechmann et al., 1999, 2000; Kohji and Matsumoto, 2000) is functionally active and induces T cell apoptosis. Complete blockade of T cell apoptosis induced by astrocytes via an antagonistic antibody of the CD95 system proves that this T cell depletion mechanism depends on the CD95/CD95 ligand system.

Since astrocytic end-feet build the glia limitans and, thus, contribute to the formation of the blood–brain barrier, CD95 ligand-expressing astrocytes might be the first line of defense in the brain against invading, activated T cells. Since CD95 ligand is upregulated on astrocytes in the inflamed (Kohji and Matsumoto, 2000; Dowling et al., 1996) and injured brain (Bechmann et al., 1999, 2000; Hirschberg et al., 1998), we propose that astrocytes contribute to an immunologic brain barrier (IBB) (Bechmann et al., 1999; Hirschberg and Schwartz, 1995) by eliminating activated, CD95 susceptible T cells from the brain. This defense mechanism may be overcome when large numbers of activated Th1 cells enter the brain and release pro-inflammatory cytokines, thus, rendering astrocytes susceptible to CD95L-induced apoptosis thereby compromising this barrier (Palma et al., 1999). In that view, the questions whether or not immune privilege is maintained, and thus, antigens located in the CNS are tolerated despite expansion of T cells specific for the respective antigens, may crucially depend on a battle between T cells and astrocytes at the glia limitans.

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