Rapid disease development in scrapie-infected mice deficient for CD40 ligand

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The inhibition of CD40–CD40L interaction-mediated signalling was suggested as a therapeutic strategy for the treatment of Alzheimer's disease. Conversely, CD40-deficient neurons were reported to be more vulnerable to stress associated with ageing as well as nerve growth factor- β and serum withdrawal. We studied the scrapie infection of CD40L-deficient (CD40L^{-/-}) mice to see whether ablation of the CD40L gene would be beneficial or detrimental in this model of a neurodegenerative amyloidosis. CD40L^{-/-} mice died on average 40 days earlier than wild-type control mice and exhibited a more pronounced vacuolation of the neuropil and an increased microglia activation. The experimental model indicates that a deficiency for CD40L is highly detrimental in prion diseases and reinforces the neuroprotective function of intact CD40–CD40L interactions. The stimulation of neuroprotective pathways may represent a possibility to delay therapeutically the disease onset in prion infections of the central nervous system.

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INTRODUCTION

CD40–CD40L interactions mediate a broad variety of immune and inflammatory responses. Besides their crucial role in T-cell-dependent humoral immunity, CD40–CD40L interactions have also been implicated in various inflammatory and autoimmune diseases, such as atherosclerosis, asthma, systemic lupus erythematosus, multiple sclerosis, graft versus host disease, experimental autoimmune encephalitis and rheumatoid arthritis (Schonbeck *et al*, 2000).

Among the central nervous system (CNS)-resident cells, CD40, a member of the tumour necrosis receptor superfamily, has been reported to be present on neurons, microglia, brain endothelial

cells and cultured human astrocytes. Its cognate ligand CD40L, a TNF- α homologue, has been found on astrocytes, endothelial cells and vascular smooth muscle cells (Abdel-Haq *et al*, 1999; Tan *et al*, 1999, 2002a).

Several lines of evidence have implicated CD40–CD40L interactions in the pathogenesis of Alzheimer's disease (AD) and led to the suggestion to target the CD40 pathway for therapeutic intervention in AD (Calingasan *et al*, 2002; Tan *et al*, 2002b). *In vitro* evidence indicated a role of CD40–CD40L in the activation of microglia by β -amyloid (A β ; Tan *et al*, 1999). In addition, a more recent study of transgenic mice that overproduced β -amyloid peptide but were deficient for CD40L, demonstrated a decreased gliosis and reduced β -amyloid plaque loads compared with controls with unimpaired CD40L signalling. The authors suggest that a reduction of CD40L-activated microglia might oppose plaque pathology, and also demonstrated a role of CD40L in amyloid-precursor-protein processing and brain-to-blood clearance of A β (Tan *et al*, 2002b).

Conversely, CD40 was reported to be constitutively expressed on neurons. CD40-deficient mice at the age of 16 months showed a pronounced neurodegeneration including gross brain abnormalities, leading to the conclusion that CD40-deficient neurons are more vulnerable to stress associated with ageing. In addition, CD40 ligation protected cultivated neurons against the adverse effects of nerve growth factor- β (NGF- β) and serum withdrawal (Tan *et al*, 2002a). Thus, a therapeutic value of disrupting CD40–CD40L signalling on β -amyloid plaque loads could be potentially offset by losing functions important for neuronal survival.

We studied the scrapie infection of CD40L^{-/-} mice in comparison to wild-type controls to address the question whether ablation of the CD40L gene would be beneficial or detrimental in this model of a neurodegenerative amyloidosis.

RESULTS AND DISCUSSION

To assess the role of CD40/CD40L interactions during a prion infection of the CNS, the intracerebral (i.c.) scrapie infection of mice deficient for CD40L was compared with similarly infected wild-type mice. The CD40L^{-/-} mice succumbed to the disease on average at 144 ± 4 days *post infectionem* (dpi), whereas the wild-type controls died at 184 ± 10 dpi (Table 1, Fig 1). However,

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Table1 | Survival times (dpi) of scrapie-infected CD40L^{-/-} and wild-type control mice

Dilution of the 10% brain homogenates used as inoculum	CD40L ^{-/-} mice (129Sv × C57B/6)	CD40L ^{+/+} mice (129Sv × C57B/6)	CD40L ^{+/+} mice (C57B/6)
10 ⁻¹	ND	ND	161 ± 2, n = 8
10 ⁻²	ND	ND	170 ± 4, n = 11
10 ⁻³	144 ± 4, n = 11	184 ± 10, n = 13 ^a	185 ± 8, n = 18 ^a
10 ⁻⁴	ND	ND	202 ± 8, n = 12

All infected animals succumbed to the disease; ND, not done.
^aNearly identical survival times of i.c.-infected 129Sv × C57B/6 and C57B/6 wild-type mice were also observed in previous experiments using the RML strain, which, like 139A, originated from the Chandler strain (Prinz et al, 2003).

Fig 1 | Survival of scrapie-infected CD40L^{-/-} mice and wild-type controls. The difference in the survival times is highly significant ($P < 0.00001$). The survival time spread was 14 days for the CD40L^{-/-} mice and 28 days for the controls.

both the duration of the symptomatic phase and the clinical appearance of the CD40L^{-/-} mice were identical to those of the controls. Thus, the CD40 deficiency leads to a very early disease onset and death compared with the control mice but does not modify the scrapie symptoms in the final stage of the infection. The highly significant ($P < 0.00001$, unpaired t -test) difference of 40 days in the survival of the two groups is equivalent to an infection of the CD40L^{-/-} mice with a 1,000 times higher infectious dose, as deduced from published dose/scrapie survival time relations for wild-type mice of similar genetic backgrounds (Klein et al, 2001) and our own observations (Table 1).

To characterize the scrapie infection of the CD40L^{-/-} mice in comparison with the wild-type controls in more detail, we studied at time point 125 dpi the gliosis, the accumulation of misfolded proteinase K-resistant prion protein (PrP^{Res}) and the scrapie-associated vacuolation of the neuropil. No significant differences were seen for activated, glial fibrillary acidic protein (GFAP)-expressing astrocytes and for the extent of PrP^{Res} deposition when analysed by the paraffin-embedded tissue (PET) blot technique or western blotting (Fig 2). However, immunohistochemical detection of activated microglia with an anti-F4/80 antibody revealed a more pronounced microgliosis in the scrapie-infected CD40L^{-/-} mice (Fig 3), which argues against a prominent role of CD40–CD40L interactions in the disease-associated microglia activation *in vivo*, which was suggested for the activation of microglia by β -amyloid *in vitro* (Tan et al, 1999).

In addition, the vacuolation of the neuropil, which is a hallmark of prion disease-associated neurodegeneration (Prusiner, 1998), was clearly more pronounced in the CD40L^{-/-} mice than in wild-type controls (Figs 2 and 3). The differences were most notably pronounced in the midbrain and the brainstem (Fig 3).

Because a preferential alteration of the inhibitory GABAergic system was demonstrated in transmissible spongiform encephalopathies (Guentchev et al, 1998), we compared the presence of parvalbumin (PV)-positive (+) neurons in CD40L^{-/-} mice with that in mock-infected and scrapie-infected wild-type controls. In cortices of CD40L^{-/-} mice, we found a significantly more severe loss of PV(+) neurons (Fig 4). These findings show that PV(+) neurons are more sensitive to degeneration in CD40L^{-/-} mice.

To substantiate the results obtained by immunohistochemistry and to gain further insight into the disease development in the CD40L^{-/-} mice compared with the wild-type controls, we performed quantitative reverse transcription (RT)–PCR for (i) lysozyme M, which is expressed by activated microglia during scrapie infections (Kopacek et al, 2000), (ii) GFAP, as a marker for the scrapie-associated astrocyte activation, and (iii) 2',5'-oligoadenylate synthetase (OAS), which is upregulated during scrapie infections and causes RNase L activation. RNase L is involved in apoptotic cell death via its nonspecific rRNA-degrading activity and may therefore directly contribute to neuronal loss in scrapie (Castelli et al, 1997; Zhou et al, 1997; Riemer et al, 2000).

In agreement with immunohistochemistry, expression levels of the microglial activation marker lysozyme M were significantly elevated in the CD40L^{-/-} mice at 125 dpi, whereas the astrocytic GFAP expression levels were similar for the CD40L-deficient mice and the controls (Fig 5). The more pronounced microglia activation in the CD40L-deficient mice may indicate an increased microglial involvement in the clearance of neuronal debris and/or remodelling of degenerated tissue. The elevated OAS mRNA levels at 125 dpi in the CD40L^{-/-} mice point to a stronger activation of apoptotic pathways, which is in agreement with the shortened survival times and increased vacuolation of the neuropil in this group. The CD40L^{-/-} mice and the controls expressed equal amounts of OAS mRNA in the terminal stage of the disease (Fig 5), which could indicate that the scrapie-induced neurodegeneration is fully established at this point.

In summary, our observations are in support of a concept that considers CD40–CD40L interactions as neuroprotective and is in agreement with previously reported functions of CD40/CD40L. The ligation of CD40 by C40L on neuronal cells stimulates the p44/42 mitogen activated protein (MAP) kinase and inhibits the

Fig 2 | PrP^{Sc} accumulation and astrocytosis in the CD40L^{-/-} mice and the wild-type controls. (A–C) (left side) PET blot analysis of the PrP^{Sc} accumulation in (A) mock-infected mice, (B) scrapie-infected wild-type controls and (C) scrapie-infected CD40L^{-/-} mice. (A–C) (right side) Immunohistochemical staining of GFAP-positive activated astrocytes. Note the more pronounced spongiosis in the CD40L^{-/-} mice (C (right side) compared with B). (D) Western blot analysis (two mice per group and time point) of proteinase K-resistant PrP^{Sc} in brain homogenates from wild-type mice (at 125 dpi (1) and at the terminal stage (2)) and from CD40L^{-/-} mice (at 125 dpi (3) and at the terminal stage (4)). Cereb, cerebellum; Cort, cortex; Hc, hippocampus; Mid, midbrain; Olf, olfactory bulb; Th, thalamus.

pro-apoptotic c-Jun N-terminal kinase pathway following NGF-β or serum withdrawal (Tan *et al*, 2002a). Further neuroprotective functions of CD40/CD40L interactions include the induction of brain-derived neurotrophic factor (BDNF) release by macrophages (Shibata *et al*, 2003). BDNF supports the survival of neurons in the CNS by activation of MAP kinases and the PtdIns-3-OH kinase (Cavanaugh *et al*, 2001; Kelly-Spratt *et al*, 2002). Hence, CD40–CD40L interactions may promote neurotrophic activities of brain-resident macrophages (microglia). It is currently unknown whether other ligands of CD40, namely C4b-binding protein (C4BP) and 70 kDa heat shock protein (hsp70), could functionally replace, at least to some degree, CD40L in this context (Becker *et al*, 2002; Brodeur *et al*, 2003). If so, it is likely that the described neuronal phenotype of CD40^{-/-} mice (Tan *et al*, 2002a) would be more severe than the phenotype of CD40L^{-/-} mice, in which the interaction of CD40 with C4BP and hsp70 is unimpaired. However, the data presented here suggest that other ligands of CD40 cannot fully compensate for the lack of neuroprotection against PrP^{res}-mediated neurotoxicity provided by CD40L.

Fig 3 | Scrapie-associated vacuolation of the neuropil in the midbrain of CD40L^{-/-} mice (A) in comparison with the age-matched control (CTR) mice (B) as revealed by HE staining. Quantification of vacuolation showed significant increases in the midbrain (F; ****P* < 0.001) and brainstem (G; **P* < 0.05) of CD40L^{-/-} mice. Immunohistochemical detection of activated microglia with an anti-F4/80 antibody revealed a more pronounced microgliosis in the scrapie-infected CD40L^{-/-} mice (C) in comparison with the wild-type controls (D). Quantification of F4/80-positive cells showed significant increases in the hippocampus (E; ***P* < 0.01) of CD40L^{-/-} mice. Scale bar, 50 μm (A–D).

In agreement with the observed massive neurodegeneration in ageing CD40^{-/-} mice (Tan *et al*, 2002a), our data suggest that missing CD40–CD40L signalling renders neurons more susceptible to the stress caused by the deposition of PrP^{res} and the subsequent glia activation (Rezaie and Lantos, 2001). The experimental model suggests that the disease development and survival in prion diseases are significantly influenced by neuroprotective factors such as CD40–CD40L, which counteract the adverse effects of the ongoing prion replication by providing functions important for neuronal survival. Hence, the stimulation of neuroprotective pathways may represent a possibility to delay therapeutically the disease onset in established prion infections of the CNS. In addition, the previously reported role of apoptosis as a mechanism in the prion disease-associated neurodegeneration (Kretzschmar *et al*, 1997; Schatzl *et al*, 1997; Williams *et al*, 1997; Jamieson *et al*, 2001; Siso *et al*, 2002) and the upregulation of OAS in scrapie-infected brain tissue (Riemer *et al*, 2000) suggest that neuroprotection could also be achieved by targeting pro-apoptotic signalling cascades or by overexpression of proteins with anti-apoptotic functions. Both possibilities are currently addressed in our laboratory.

Fig 4 | Loss of PV-positive GABAergic neurons. PV-positive neurons were stained in the cortices of mock-infected wild-type mice (A), of scrapie-infected wild-type mice (B) and of CD40L^{-/-} mice (C) with a PV-specific monoclonal antibody. Quantification of PV-positive neurons showed a significant decrease (* $P < 0.01$) in the CD40L^{-/-} mice compared with the wild-type controls (D). Scale bar, 50 μ m (A–C).

Fig 5 | Relative lysozyme, OAS and GFAP mRNA levels in individual CD40L^{-/-} mice compared with the wild-type controls (two mice per group and time point). All mRNA expression levels were determined in triplicate (mean fold increase $\pm$ s.e.). The CD40L-deficient mice show a 10- to 11-fold increased lysozyme and a 4- to 7-fold higher OAS expression, respectively, at 125 dpi. Only the lysozyme expression was still slightly elevated in the terminal stage of the disease. GFAP mRNA levels are unaltered in the CD40L^{-/-} mice compared with the controls.

As far as strategies for the therapy of AD are concerned, it remains to be seen whether the neuroprotective functions of intact CD40–CD40L interactions outweigh the described beneficial influence of a CD40L deficiency on the β -amyloid plaque load and the associated gliosis observed in a murine AD model.

METHODS

Animals and scrapie infections. In all, 5-week-old, female CD40L-deficient 129Sv $\times$ C57B/6 mice, age- and sex-matched wild-type 129Sv $\times$ C57B/6 (The Jackson Laboratory, USA) controls and wild-type C57B/6 mice were intracerebrally inoculated with 20 μ l of diluted 10% brain homogenates prepared from mouse infected with the scrapie strain 139A (courtesy of R.H. Kimberlin, Edinburgh, UK). All infections with a given dilution of the brain homogenate were carried out on the same day using the same aliquot as inoculum. Animals were monitored twice per week for the development of clinical signs of the disease, which typically include a poor coat condition, a hunched posture, the development of a hindleg paralysis, weight loss and behavioural changes. Mice were killed at 125 dpi and at the terminal stage of the disease. The terminal stage was reached when the development of the scrapie symptoms indicated that the animals would die, if not killed, within the next 72 h. One hemisphere was fixed in formaldehyde for morphological analysis, and the other hemisphere was frozen and used for immunohistochemistry.

Histology and immunohistochemistry. Serial sagittal 8 μ m sections were cut from the formaldehyde-fixed and frozen hemispheres. Haematoxylin and eosin (HE) staining was performed according to standard protocols. All immunohistochemistry was performed according to previously published procedures (Guentchev *et al*, 1998; Jeffrey *et al*, 1998). Antibodies used were a polyclonal anti-GFAP antiserum (DAKO), a monoclonal antibody against the F4/80 antigen (Serotec) and a monoclonal antibody against PV (Parv-19, Sigma). The prion protein was detected using the anti-PrP monoclonal antibody 6H4 (Prionics).

Detection of misfolded proteinase K-resistant prion protein by PET blot and western blot analysis. The PET blot analysis was carried out as described previously (Schulz-Schaeffer *et al*, 2000). Briefly, 6 μ m paraffin sections of formaldehyde-fixed brain tissues were transferred onto a nitrocellulose membrane and, after dewaxing, treated with proteinase K for digestion of normal PrP^C. The remaining membrane-bound protein was subsequently denatured with 3 M guanidine isothiocyanate. The actual immunodetection was performed with the anti-PrP 6H4 antibody followed by incubation with an alkaline phosphatase-linked anti-mouse immunoglobulin antiserum. The final staining was carried out with NBT/BCIP. For the western blot analysis of proteinase K-resistant PrP^{res}, brain homogenates from two animals per group were subjected to proteinase K digests as described previously (Baier *et al*, 2003). The subsequent detection of PrP^{res} was performed using the anti-PrP antibody 6H4.

Histological evaluation. To detect differences in the degree of spongiform lesions, PrP deposition, microgliosis and astrocytosis, a quantitative scoring system was applied. The entire brain section in each specimen was examined and the degree of lesion was graded from 0 to 3+ according to the severity of damage. Examined areas were the cortex, hippocampus, brainstem, cerebellum and the olfactory bulb. Differences between CD40L^{-/-} mice and the wild-type controls were evaluated by independent scoring of all tissue samples by three investigators without prior knowledge of the group to which the mice belonged.

Quantification of PV-positive neurons. Cell numbers per area were determined by counting (Aherne, 1975). Only those sections in which the PV(+) neurons were unmistakably identifiable were included. Immunoreactive neurons were counted in ten fields

with a $\times 40$ objective using a graticule (Leitz). The mean number of immunoreactive cells was calculated and used for statistical analysis (*t*-test).

TaqMan PCR for the quantification of mRNA levels. RNAs were reverse transcribed using the First-Strand cDNA Synthesis Kit (Amersham). Gene expression levels from two or three mice per group were subsequently determined by TaqMan real-time PCR using a GeneAmp 5,700 Sequence Detection system (Perkin-Elmer) and the SYBR Green PCR Kit (Qiagen). Sequences of the PCR primers (forward and reverse, respectively) used for quantification were lysozyme (5'-GGG AAA CAG CAG TCG TG TG-3', 5'-CTG GGA ACA TCC TCT CAA GG-3'), 2',5'-oligoadenylate synthetase (OAS) (5'-GGT CTC TGA GCT TCA AGC TGA G-3', 5'-TAC TGT GGA GGC AAT GGC TTC-3') and GFAP (5'-GCG GGA GTC GGC CAG TTA CC-3', 5'-GAC CTC ACC ATC CCG CAT CT-3'). To ensure equivalence of the samples, results were normalized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5'-GAC CTC ACC ATC CCG CAT CT-3', 5'-GCG GGA GTC GGC CAG TTA CC-3').

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