Neutralization of TNFSF10 ameliorates functional outcome in a murine model of Alzheimer’s disease

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Alzheimer’s disease is one of the most common causes of death worldwide, with poor treatment options. A tissue landmark of Alzheimer’s disease is accumulation of the anomalous protein amyloid-β in specific brain areas. Whether inflammation is an effect of amyloid-β on the Alzheimer’s disease brain, or rather it represents a cause for formation of amyloid plaques and intracellular tangles remains a subject of debate. TNFSF10, a proapoptotic cytokine of the TNF superfamily, is a mediator of amyloid-β neurotoxicity. Here, we demonstrate that blocking TNFSF10 by administration of a neutralizing monoclonal antibody could attenuate the amyloid-β-induced neurotoxicity in a triple transgenic mouse model of Alzheimer's disease (3xTg-AD). The effects of TNFSF10 neutralization on either cognitive parameters, as well as on the expression of TNFSF10, amyloid-β, inflammatory mediators and GFAP were studied in the hippocampus of 3xTg-AD mice. Treatment with the TNFSF10 neutralizing antibody resulted in dramatic improvement of cognitive parameters, as assessed by the Morris water maze test and the novel object recognition test. These results were correlated with decreased protein expression of TNFSF10, amyloid-β, inflammatory mediators and GFAP in the hippocampus. Finally, neutralization of TNFSF10 results in functional improvement and restrained immune/inflammatory response in the brain of 3xTg-AD mice in vivo. Thus, it is plausible to regard the TNFSF10 system as a potential target for efficacious treatment of amyloid-related disorders.

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Introduction

Alzheimer’s disease is one of the leading causes of death worldwide. So far, only palliative treatments are available for Alzheimer’s disease, which yield poor benefits to patients suffering from this disorder (Rafii, 2013). Progression of cognitive impairment typical of Alzheimer’s disease is associated with accumulation of the anomalous, neurotoxic protein amyloid-β in various brain areas, with special regard to the hippocampus (Querfurth and LaFerla, 2010).

Amyloid-β, which forms aggregates that accumulate as the typical brain plaques, is regarded as a pivotal factor in the pathogenesis of Alzheimer’s disease (Selkoe, 2000), along with the hyperphosphorylation of the tau protein, which in turn is deposited inside neurons under the form of neurofibrillary tangles (Rosenmann, 2013).

Nevertheless, Alzheimer’s disease pathogenesis is thought to be also substantially contributed to by different factors, including genetics (Bettens et al., 2013), free radicals (Bonda et al., 2010), excitotoxicity (Hynd et al., 2004), and inflammation (Amor et al., 2014). Neuroinflammation occurs concomitantly with deposition of amyloid-β in the brain tissue (Weiner and Frenkel, 2006), and is associated with both gliaosis (Kamphuis et al., 2014) and release of various mediators of the inflammatory/immune response (Chakraborty et al., 2013), including cytokines (Griffin, 2013).

It is also reported that both injured neurons and activated glia express and release substantial amounts of proinflammatory/proapoptotic cytokines (Deverman and Patterson, 2009), which amplify and eventually exacerbate the on-going neurodegenerative process (Griffiths et al., 2009).

We have previously shown that tumor necrosis factor (ligand) superfamily, member 10 (TNFSF10; previously known as TRAIL), a proapoptotic cytokine (Almasan and Ashkenazi, 2003), is also released by human neural cells challenged with amyloid-β in vitro (Cantarella et al., 2003), and—in addition—by bacterial lipopolysaccharide-activated glia (Cantarella et al., 2007). Interestingly, TNFSF10 immunoreactivity has been detected nearby amyloid-β plaques in post-mortem human Alzheimer’s disease brains (Uberti et al., 2004).

To exert its apoptotic effects, TNFSF10 binds to two death domain receptors, TNFRSF10A (previously known as DR4) and TNFRSF10B (previously known as DR5), which transduce a caspase-dependent death signal (LeBlanc and Ashkenazi, 2003). Neutralization of TNFSF10 is associated with rescue from death of neurons challenged with amyloid-β in vitro (Cantarella et al., 2003), as well as with significant functional recovery in animal models of nervous tissue injury in vivo (Cantarella et al., 2010).

Based on these findings, we verified whether TNFSF10 could play a pathophysiological role in Alzheimer’s disease, and whether its neutralization might bring about improvement of cognitive impairment.

To do so, the expression of TNFSF10 and its receptors, as well as cognitive parameters, were studied in the triple transgenic mouse model of Alzheimer’s disease (3xTg-AD), a strain homozygous for the Psen1 mutation and homozygous for the co-injected APPSwe and tauP301L transgenes, which presents an age-dependent increase of amyloid-β oligomer accumulation, extracellular plaques in the cortex and hippocampus, and tau pathology paralleled by learning and memory impairment (Oddo et al., 2003). To corroborate the above hypothesis of a role of TNFSF10 in the cognitive impairment of 3xTg-AD mice, the effects of chronic anti-TNFSF10 treatment on inflammation and cognitive function were next evaluated in these animals.

Materials and methods

The TNFSF10-neutralizing antibody (purified rat anti-mouse CD253), and the vehicle (purified rat IgG2α isotype control) were obtained from BD Biosciences. All other compounds were of the highest commercial grade available.

Animals

Male 3xTg-AD mice [B6129-Psen11Mtm1Pmgmpg (APPSwe, tauP301L)1Lfa/J] and wild-type mice (B6129SF2/J) were purchased from Jackson Laboratories. The 3xTg-AD, overexpressing mutant APP (APPSwe), PSEN1 (PS1M146V), and hyperphosphorylated tau (tauP301L), were originally generated by co-injecting two independent transgene constructs encoding human APPSwe and tauP301L (4R/0N) (controller by murine Thy1.2 regulatory elements) in single-cell embryos harvested from mutant homozygous PS1M146V knock-in mice. Wild-type mice of mixed genetic background 129/ C57BL6 were used as controls. These mice have been characterized and described by Oddo et al. (2003). The animals were maintained on a 12-h light/dark cycle in temperature and humidity-controlled rooms, and food and water were available ad libitum. All experiments were carried out according to the recommendations of Institutional Animal Care and Use Committee (IACUC).

Drug administration and experimental groups

Twenty-two 3xTg-AD and 22 wild-type mice were enrolled at 3 months of age and four study groups were used: (i) wild-type plus vehicle; (ii) wild-type plus TNFSF10-neutralizing antibody; (iii) 3xTg-AD plus vehicle; and (iv) 3xTg-AD plus TNFSF10-neutralizing antibody. Animals were treated with TNFSF10-neutralizing antibody (10 μg/mouse; intraperitoneally) or vehicle (10 μg/mouse; intraperitoneally) (mouse weight = 25 ± 5 g) twice a month and sacrificed at 9 months of age.

Protein extraction

Tissues were lysed in a lysis buffer containing 150 manacle, 50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 1 mM Na3VO4, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM acid phenyl-methyl-sulphonyl-fluoride, 5 μg/ml aprotinin, 2 μg/ml
leucine, 1 μg/ml peptatin, 10% glycerol, and 0.2% Triton® X-100. The homogenates were then centrifuged at 14 000 rpm for 10 min at 4°C. The protein concentration of the supernatant was determined by the Bradford method (Bradford, 1976).

**Western blot analysis**

Equal amounts of protein (50 μg) were subjected to SDS-PAGE on 8 and 12% gels and then transferred onto Hybond ECL nitrocellulose membranes (Amersham Life Science). The membranes were blocked with 5% milk in PBS-Triton® X-100 and then incubated overnight at 4°C with rabbit anti-TNFRSF10B polyclonal antibody (Abcam), rabbit anti-TNFRSF10 polyclonal antibody (Abcam), rabbit anti-beta-amyloid1-42 polyclonal antibody (Millipore Corporation), rabbit anti-Cox-2 polyclonal antibody (Santa Cruz Biotechnology), rabbit anti-IL1B polyclonal antibody (Santa Cruz Biotechnology), mouse anti-GFAP monoclonal antibody (Santa Cruz Biotechnology); and horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody (Cell Signaling Technology), or rabbit anti-NOS2 polyclonal antibody (Santa Cruz Biotechnology) and mouse anti-β-amyloid 1–42 polyclonal antibody (Abcam), rabbit anti-TNFRSF10 polyclonal antibody (1:100; Abcam), or a rabbit anti-IL1B polyclonal antibody (1:200; Santa Cruz Biotechnology). The membranes were labelled with peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody (Amersham Life Science). β-Tubulin (Santa Cruz Biotechnology) was used as an internal control to validate the right amount of protein loaded in the gels. Detection was performed by means of ECL chemiluminescence assay (Amersham Life Science).

**Immunohistochemistry**

Tissue specimens were fixed overnight in 10% neutral buffered formalin (Bio-Optica). After overnight washing they were dehydrated in graded ethanol and paraffin-embedded taking care to preserve their anatomical orientation. Sections were then cut in the coronal plane and 5-μm thick sections were then obtained by routine procedures, mounted on silanized glass slides and air-dried.

For immunohistochemistry, first endogenous peroxidase activity was quenched with 3% H2O2 for 10 min. Non-specific antibody binding was blocked with normal horse/goat serum [diluted 1:20 in PBS, 0.1% bovine serum albumin (BSA)]. Sections were irradiated (5 min × 3) in capped polypropylene slide-holders with citrate buffer (pH 6.0) using a microwave oven (750 W) to unmask antigen sites.

The following primary antibodies were used: a rabbit anti-TNFRSF10B polyclonal antibody (1:100) (Abcam), or a rabbit anti-TNFRSF10 polyclonal antibody (1:100; Abcam), or a rabbit anti-Cox-2 polyclonal antibody (1:200; Santa Cruz Biotechnology), or a rabbit anti-NOS2 polyclonal antibody (1:200; Santa Cruz Biotechnology), or a rabbit anti-IL1B polyclonal antibody (1:100; Santa Cruz Biotechnology). Antibodies were applied directly onto sections before overnight slide incubation (4°C) in a humid chamber. Immune complexes were treated with a biotinylated link antibody and detected with peroxidase-labelled streptavidin, both incubated for 10 min at room temperature (LSAB + System-HRP, Dako Italia).

The immunoreaction was visualized by incubating sections in 0.1% 3,3′-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB; Substrate Chromogen System, Dako) for 4 min. Sections were then lightly counterstained with Mayer’s haematoxylin (Histolab Products AB), mounted in GVA mount (Zymed, Laboratories) and observed using an Axiosplan light microscope (Zeiss).

Positive controls consisted of tissue specimens with known antigen positivity. Negative control sections were processed using the same protocol as the experimental slides except that they were incubated with normal rabbit serum.

**Evaluation of immunostaining**

To quantify immunohistochemical staining, 10 sections/sample were analysed in stepwise fashion as a series of consecutive fields with a × 40 magnification; the stained area was expressed as pixels/field. Randomly selected fields from each section were analysed and the per cent area staining for TNFRSF10 and TNFRSF10B was calculated using AxioVision rel. 4.8.2 image analysis software and an AxioVision 4 Module AutoMeasure (Zeiss) to quantify the level of immunolabelling in each field. Values from all consecutive images of each biopsy were averaged. Digital pictures were taken using an Axioacam camera (Zeiss).

All variables were normally distributed. Comparisons between means were tested by ANOVA. P-values < 0.05 were considered significant.

**Behavioural studies**

For behavioural studies, we used 11 3xTg-AD mice treated with vehicle, and 11 mice for each of the following conditions: 3xTg-AD mice treated with anti-TNFRSF10, wild-type mice treated with vehicle, wild-type mice treated with anti-TNFRSF10. Morris water maze experiments were performed as described (Puzzo et al., 2012; Palmeri et al., 2013). The maze consisted of a white tank filled with water (24–25°C) made opaque by the addition of non-toxic white paint. Spatial cues were presented on the walls of the testing room. An underwater platform measuring 10 × 10 cm was placed in one of the maze quadrants and left there throughout the tests. Mice were trained for 3 days in two daily sessions each consisting of three 1-min trials, in which mice started from a different, randomly chosen quadrant; sessions were held 4 h apart, so that mice had to rely on long-term memory of platform location. Time taken to reach the hidden submerged platform (latency) was recorded. The fourth day the platform was removed and spatial memory retention was tested in four probe trials. The maze was divided into four quadrants, the target quadrant (i.e. the one previously containing the platform), and three non-target quadrants (adjacent left, adjacent right, and opposite quadrant). The per cent time spent in each quadrant was recorded and analysed with a video tracking system (Netsense srl). After the probe trials, on the fifth and sixth day, visual, motor, and motivation skills were tested in two sessions/day (each consisting of three 1-min trials) by measuring the time taken to reach a visible platform (randomly positioned in a different place each time) marked with a green flag.

Object recognition testing was performed as previously described (Puzzo et al., 2013). Two days before training, mice were handled gently for 5 min and then allowed to familiarize with the apparatus (a plastic box 50 cm long, 35 cm wide, and 15 cm high) for 10 min/day. The object recognition test consisted of two 10-min trials, one per day. This protracted exposure allowed the animals to learn the task. In the first trial (T1), two identical objects were placed in the central part of the box, equally distant from the perimeter.
Each mouse was placed in the apparatus and allowed to explore them. Exploration was defined as the mouse pointing its nose toward the object from a distance of no more than 2 cm (as marked by a reference circle). The mouse was then returned to its cage. The second trial (T2) was performed 24 h later to test memory retention. Mice were presented with two objects, a ‘familiar’ (i.e. the one used for T1) and a ‘novel’ object. The latter object was placed on the left or the right side of the box in a randomly but balanced manner, to minimize potential biases due to a preference for particular locations or objects. To avoid olfactory cues, the objects and the apparatus were cleaned with 70% ethanol after each trial. The following parameters were evaluated: (i) time of exploration of the two objects expressed as % exploration of the novel and % exploration of the familiar object; (ii) discrimination (D) index calculated as ‘exploration of novel object minus exploration of familiar object / total exploration time’; (iii) latency to first approach to novel object; and (iv) total exploration time (% exploration of the novel + % exploration of the familiar object).

Immunofluorescence

For double immunofluorescence brain sections were incubated overnight at 4°C with the following primary antisera: rabbit anti-NOS2 polyclonal antibody (1:200; Santa Cruz Biotechnology) and mouse anti-GFAP monoclonal antibody (1:300; Cell Signaling Technology). After 24 h of primary antibody incubation, the brain sections processed for double immunofluorescence were incubated in a mixture of the fluorescent-labelled secondary antibodies [Alexa Fluor® 488 goat anti-rabbit IgG (1:200; Life Technologies); or goat anti-mouse IgG-TR (1:200; Santa Cruz Biotechnology)].

For brain localization of TNFSF10-neutralizing antibody or vehicle, animals were treated with Alexa Fluor® 555 rat anti-mouse CD253 (BD Biosciences) or Alexa Fluor® 555 rat IgG2aa Isotype Control (BD Biosciences) (10μg/mouse; intraperitoneally) and sacrificed after 10 days. To detect brain localization of TNFSF10-neutralizing antibody or vehicle on 6 months treated animals, immunofluorescence were incubated in a mixture of the fluorescent-labelled secondary antibodies [Alexa Fluor® 488 goat anti-rabbit IgG (1:200; Life Technologies); or goat anti-mouse IgG-TR (1:200; Santa Cruz Biotechnology)].

All images were observed using a laser scanning confocal microscope (Zeiss LSM 700).

Statistics

All experiments were blind with respect to treatment. Data were expressed as mean ± standard error mean (SEM). For behavioural studies, statistical analysis was performed using software (System). In particular, we used two-way ANOVA with repeated measures (for time and treatment i.e. between and within subjects) for latency, two samples t-test to compare the time spent in target quadrant by each group and to analyse the difference in exploration time of the familiar versus the novel object, and one sample t-test to compare the D index with zero, to establish whether animals recognized the familiar object in T2. The level of significance was set at P < 0.05.

Results

Immunoneutralization of TNFSF10 reduces expression of its TNFRSF10B receptor, TNFSF10 itself and amyloid-β

To verify the biological validity of the hypothesis that the potent proapoptotic cytokine TNFSF10 is implicated in neuronal death in amyloid-mediated toxicity, 3-month-old 3xTg-AD mice were treated intraperitoneally twice a month for 6 months with a TNFSF10 neutralizing monoclonal antibody. The expression of TNFSF10 and its TNFRSF10B (DR5) death receptor was then studied by western blot in protein homogenates from the hippocampus of each animal. The increase in expression of TNFSF10 and its TNFRSF10B receptor found in 3xTg hippocampus was significantly reduced in animals previously treated with anti-TNFSF10 (Fig. 1A and B). Similar results were obtained by immunohistochemical analysis, which showed that the expression of both TNFSF10 and its TNFRSF10B receptor was significantly increased in the hippocampus of untreated 3xTg-AD mice compared to wild-type animals (Fig. 1C and D). Such expression was reduced after treatment with the anti-TNFSF10 antibody in 3xTg-AD mice (Fig. 1C and D). Immunohistochemistry also demonstrated that the number of TNFSF10 and TNFRSF10B immunoreactive cells was higher in 3xTg-AD mice compared with wild-type animals (Fig. 1C and D), whereas it decreased in mice receiving the anti-TNFSF10 antibody (Fig. 1C and D), in all the areas studied (CA1, CA2 and CA3).

Hippocampal sections of wild-type mice receiving either vehicle or the anti-TNFSF10 treatment exhibited weak TNFSF10 and TNFRSF10B immuno-expression in scattered glial cells in the CA1 field; on the other hand, increased TNFSF10 and TNFRSF10B immuno-expression was demonstrated in the cytoplasm of pyramidal neurons in the CA1 field of untreated 3xTg animals, whereas sections from 3xTg-AD mice treated with anti-TNFSF10 showed significantly decreased TNFSF10 and TNFRSF10B immuno-expression in the same area (Fig. 1C and D).

As TNFSF10 is a mediator of amyloid toxicity and the increase of amyloid-β is the major hallmark of 3xTg-AD mice, we evaluated the expression of amyloid-β1-42 by western blot analysis in the hippocampus of the same groups of animals. Results indicated that the hippocampal expression of amyloid-β in 3xTg-AD animals that underwent the anti-TNFSF10 treatment was significantly reduced (Fig. 2).

Neutralization of TNFSF10 restores cognitive behaviour in 3xTg-AD mice

We assessed the effect of the anti-TNFSF10 treatment on cognition in 3xTg-AD mice. Mice were first tested for
Figure 1 Immunoneutralization of TNFSF10 reduces the expression of the TNFRSF10B receptor and TNFSF10 itself in the hippocampus of 3xTg AD mice. (A) Western blot analysis of the TNFRSF10B, and TNFSF10 proteins in the hippocampus (Hp) of 3xTg-AD mice treated for 6 months with an anti-TNFSF10 monoclonal antibody or vehicle. WT = wild-type mice; AD = 3xTg-AD mice and the corresponding (B) densitometric analysis. *,# $P < 0.05$ versus respective controls (one-way ANOVA, followed by a Duncan’s test for paired differences). (C) TNFRSF10B and TNFSF10 (D) immunohistochemical expression in the whole hippocampus (top) and in the CA1 hippocampal area (bottom) of 3xTg-AD (AD) and their corresponding wild-type (WT) mice treated for 6 months with an anti-TNFSF10 monoclonal antibody or vehicle. Scale bar = 100 μm. The bottom histograms represent per cent count of immunoreactive cells (left), and the relative immunostaining intensity (right) in the different experimental groups; *,# $P < 0.05$ versus respective controls (one-way ANOVA, followed by a Duncan’s test for paired differences). Vertical bars are means ± SEM.
spatial learning and reference memory with the Morris water maze, a widely used test known to require hippocampal function (Schenk and Morris, 1985).

During the Morris water maze training, mice were requested to find a hidden platform beneath the surface of the water. As previously demonstrated (McKee et al., 2008), and in parallel with the increased TNFSF10 and TNFRSF10B receptor expression in their hippocampus, untreated 3xTg-AD mice showed significant impairment in the ability to learn this task compared to age-matched wild-type mice. Indeed, Alzheimer’s disease mice took longer to find the hidden platform, thus confirming the spatial learning impairment [overall latency: 34.7 ± 2.77s versus 20.4 ± 3.66s in 3xTg-AD and wild-type mice, respectively; \( F(1,19) = 16.65, P = 0.001 \)] that was rescued by previous treatment with anti-TNFSF10 [overall latency: 22.5 ± 2.42s; \( F(1,20) = 0.42, P = 0.522 \) compared with vehicle-treated wild-type]. Anti-TNFSF10 did not affect performance in wild-type mice [overall latency: 20.8 ± 1.91s; \( F(1,20) = 0.02, P = 0.883 \) (Fig. 3A).

After the sixth hidden-platform session, we assessed reference memory with the probe trial. The platform was removed from the pool and animals were allowed 60s to search for it. The maze was virtually divided in four areas, and the amount of time spent in each quadrant of the maze was calculated. Performance was evaluated comparing the per cent time spent in the target quadrant, where the platform had been located during training, and in the other quadrants. Vehicle-treated 3xTg-AD mice showed an impairment of reference memory, as shown by the fact that they spent less time in the target quadrant [32.79 ± 5.5% versus 58.36 ± 6.24%; \( t(19) = 7.04, P < 0.0001 \)] compared with vehicle-treated wild-type mice. This reference memory impairment was rescued by anti-TNFSF10 treatment that lead 3xTg-AD mice to normally perform [54.09 ± 6.38%; \( t(20) = 1.12, P = 0.276 \) versus vehicle-treated wild-type mice], as well as wild-type animals treated with anti-TNFSF10 [55.13 ± 5.06%; \( t(20) = 3.2, P = 0.358 \) (Fig. 3B). A visible platform trial performed after the probe trials did not reveal any difference in the time needed to reach the platform among the four groups [\( F(3,39) = 0.22, P = 0.88 \); Fig. 3C].

Next, we studied recognition memory, a task based on the natural tendency of rodents to explore unfamiliar objects, which depends in part on hippocampus integrity (Broadbent et al., 2010; Barker and Warburton, 2011). The object recognition test included two sessions. During the first day (T1), mice were allowed to explore two identical objects; after 24 h (T2) mice were kept in the arena containing the same object as in T1 and a new object. We first measured the time of exploration for the familiar and the novel object. 3xTg-AD mice treated with vehicle displayed an impairment of memory, as they spent almost the same time exploring the familiar and the novel object [44.45 ± 4.47% versus 55.43 ± 4.47% exploration time spent with familiar versus novel object; \( t(18) = 1.48, P = 0.156 \)]. However, treatment with anti-TNFSF10 restored recognition memory, as demonstrated by the fact that animals spent more time exploring the novel object [30.53 ± 2.22% versus 69.46 ± 2.22% exploration time spent with familiar versus novel object; \( t(20) = 10.95, P < 0.0001 \)]. Wild-type mice treated with vehicle or anti-TNFSF10 showed a good recognition memory [vehicle: 34.31 ± 3.88% versus 65.68 ± 3.88% exploration time spent with familiar versus novel object; \( t(20) = 5.06, P < 0.0001 \); anti-TNFSF10: 31.11 ± 4.04% versus 68.88 ± 4.04% exploration time spent with familiar versus novel object; \( t(20) = 5.85, P < 0.0001 \) (Fig. 4A).

The discrimination index (D), calculated as ‘novel object exploration – familiar object exploration / total exploration time’ confirmed that D was different in 3xTg-AD mice treated with vehicle and anti-TNFSF10 [\( t(19) = 2.44, P = 0.024 \) and that 3xTg-AD mice were not able to learn
because D did not significantly differ from zero $[t(9) = 1.04, P = 0.322]$. A statistically significant difference in the D index was not detected between wild-type mice treated with vehicle or anti-TNFSF10, both able to learn the task $[\text{wild-type + vehicle: } t(10) = 3.59, P = 0.005; \text{wild-type + anti-TNFSF10: } t(10) = 4.14, P = 0.002; \text{Fig. 4B}]$. The four groups of mice did not show differences in the latency to first approach the novel object $[F(3,39) = 0.15, P = 0.929; \text{Fig. 4C}]$ or the total exploration time $[F(3,39) = 0.12, P = 0.944; \text{Fig. 4D}]$.

Thus, neutralization of TNFSF10 rescued spatial learning, reference memory and recognition memory in 3xTg-AD mice.

**Treatment with anti-TNFSF10 antibody reduces expression of inflammatory molecules and kinases**

To establish whether molecular and functional changes observed could somehow be related to a neuroinflammatory process in the 3xTg-AD mice brain, and to explore the influence of TNFSF10 neutralization on a set of inflammatory molecules, either PTGS2 (previously known as COX2), NOS2 (previously known as iNOS) and IL1B were measured by means of western blot analysis of hippocampal lysates from the same groups of animals.

Western blot analysis revealed significantly increased expression of the three proteins in the lysates from the hippocampus of untreated 3xTg-AD mice compared to that of wild-type animals (Fig. 5A and B). Such elevated expression was significantly decreased after treatment with the anti-TNFSF10 antibody (Fig. 5A and B).

Immunohistochemical analysis of CA1 area in the hippocampus revealed a significant increase in expression of inflammatory molecules in Alzheimer’s disease compared to wild-type tissues (Fig. 5C). Consistently, the number of neuronal cells positive for inflammatory molecules (PTGS2, NOS2 and IL1B) in the hippocampus of 3xTg-AD mice was higher compared to that of wild-type animals (Fig. 5C). The percentage of immune-stained cells was decreased in 3xTg-AD mice treated with anti-TNFSF10 (Fig. 5C).

**Anti-TNFSF10 treatment of 3xTg-AD mice is associated with reduction of gliosis**

As gliosis is a common feature of neurodegenerative processes and is also associated with inflammation (Kamphuis et al., 2014), we verified whether glial component of brain cells was increased in 3xTg mice, and whether the expression of its specific marker GFAP could eventually change in various treatment groups.

GFAP expression was significantly increased in the hippocampus of untreated 3xTg-AD mice compared to wild-type animals. On the other hand, the expression of GFAP was dramatically decreased in animals that received the anti-TNFSF10 treatment (Fig. 6A and B).

Immunohistochemical data were consistent with those obtained by western blot and eventually revealed that GFAP positive cells were significantly decreased in animals.
treated with anti-TNFSF10 serum when compared with untreated, transgenic mice.

In addition, western blot analysis also indicated that the substantial NOS2 expression detected in 3xTg-AD mice was reduced after the anti-TNFSF10 treatment (Fig. 6A and 6B). Finally, image merging by confocal microscopy revealed that GFAP immunoreactivity is overlapping NOS2, indicating that neutralization of TNFSF10 results in attenuation of activity of glial cells in the hippocampus of 3xTg-AD (Fig. 6C).

Kinetics of the anti-TNFSF10 antibody

It is known that the half-life of IgG2α is ~21 days (Mould and Sweeney, 2007). On this basis, to create conditions for steady state of its plasma levels, we administered the antibody every 15 days for the whole duration of our study.

To verify that during its distribution after injection the anti-TNFSF10 antibody effectively reached the brain, immunofluorescence was performed in histological specimens from animals sacrificed at the end of treatment, using a secondary fluorescent anti-rat IgG. Fluorescence was detected in the hippocampus of all samples examined (Fig. 7A).

Moreover, to verify effective blood–brain barrier crossing of the anti-TNFSF10 antibody administered intraperitoneally, the fluorescent form of the rat anti-mouse TNFSF10 antibody was injected in naive 3-month-old wild-type and Alzheimer’s disease mice. Immunofluorescence analysis of data performed 10 days after, revealed the presence of...
Figure 5 Anti-TNFSF10 treatment attenuates expression of inflammatory molecules in the hippocampus of 3xTg AD mice.

(A) Western blot analysis of cyclooxygenase 2 (COX2, now known as PTGS2), inducible nitric oxide synthase (iNOS, now known as NOS2), and interleukin-1β (IL1B) proteins in the hippocampus of 3xTg-AD mice treated for 6 months with an anti-TNFSF10 monoclonal antibody or vehicle. WT = wild-type mice; AD = 3xTg-AD mice and the corresponding (B) densitometric analysis. *#$p < 0.05 versus respective controls (one-way ANOVA, followed by a Duncan's test for paired differences). Vertical bars are means ± SEM. (C) PTGS2, NOS2 and IL1B immunohistochemical expression in the CA1 hippocampal area of 3xTg-AD and their corresponding wild-type mice treated for 6 months with an anti-TNFSF10 monoclonal antibody. The bottom histograms represent, per cent count of immunoreactive cells (left), and the relative immunostaining intensity (right) in the different experimental groups: *#$p < 0.05 versus respective controls (one-way ANOVA, followed by a Duncan's test for paired differences). Vertical bars are means ± SEM. Scale bar = 100 μm.
Figure 6  Anti-TNFSF10 treatment reduces gliosis in the hippocampus of 3xTg-AD mice. (A) Western blot analysis of GFAP, iNOS, and β-Tubulin proteins in the hippocampus of 3xTg-AD mice treated for 6 months with an anti-TNFSF10 monoclonal antibody or vehicle. WT = wild-type mice; AD = 3xTg-AD mice and its corresponding (B) densitometric analysis. *, # P < 0.05 versus respective controls (one-way ANOVA, followed by a Duncan’s test for paired differences). (C) Representative immunofluorescence microscopy imaging showing co-localization of GFAP and NOS2 in glial cells of hippocampal sections from 3xTg-AD mice treated for 6 months with an anti-TNFSF10 monoclonal antibody or vehicle. GFAP and NOS2 (iNOS) were detected by means of double immunostaining and then the respective images were merged. e, f, g, h are the sectors shown in E, F, G, H respectively. i, j, k, l are the sectors shown in I, J, K, L respectively. I shows merge of I’ and I” images. J shows merge of J’ and J” images. K shows merge of K’ and K” images. L shows merge of L’ and L” images. Scale bars: A, B, C, D = 100 μm; E, F, G, H = 20 μm; I, J, K, L = 10 μm.
Figure 7  Immunofluorescence analysis of rat IgG isotype control and anti-TNFSF10 in the brain of either wild-type or Alzheimer's disease mice. (A) Representative immunofluorescence imaging of the hippocampus in brain slices from 9-month-old 3xTg-AD mice treated for 6 months with a rat anti-mouse TNFSF10 monoclonal antibody, using a fluorescent secondary anti rat IgG. WT = wild-type mice; AD = 3xTg-AD mice. e, f, g, h are the sectors shown in E, F, G, H, respectively. Scale bars: A, B, C, D = 100 μm; E, F, G, H = 20 μm.

(B) Representative immunofluorescence imaging of the hippocampus in brain slices from 3-month-old 3xTg-AD mice sacrificed 10 days after intraperitoneal treatment (10 μg/animal) with a fluorescent rat anti-mouse TNFSF10 monoclonal antibody. e, f, g, h, i, j, k, l are the sectors shown in E, F, G, H, I, J, K, L, respectively. Scale bars: A, B, C, D = 100 μm; E, F, G, H, I, J, K, L = 20 μm.
the antibody in the brain of both wild-type and Alzheimer’s disease mice (Fig. 7B).

**Discussion**

In this work, for the first time, we have demonstrated that neutralization of the proapoptotic cytokine TNFSF10 results in substantial functional recovery from progressive cognitive impairment, related to improvement of inflammatory marker parameters in the brain and, specifically in the hippocampus.

The study of the expression of TNFSF10 and its TNFRSF10B receptor in brain areas of transgenic 3xTg-AD mice showed that it is increased in the hippocampus. Immunohistochemical analysis confirmed an increased specific immunoreactivity of the two molecules in that area in all experimental conditions. In fact, while it has been reported that TNFSF10 expression is absent in normal brain, the latter, nevertheless, constitutively expresses its TNFRSF10B receptor to a certain extent (Almasan and Ashkenazi, 2003).

Also, the appearance of the expression of TNFSF10 and TNFRSF10B in the brain of 3xTg-AD mice was time-related, consistent with the report that the 3xTg-AD mouse develops progressive impairment of cognitive behaviour, paralleled by the appearance of amyloid plaques and neurofibrillar tangles in the brain (Oddo et al., 2003). In fact, as a confirmation of these data, we found that 3xTg-AD mouse show a higher expression of the toxic fragment of amyloid-β1-42.

In previous work, Cantarella et al. (2003, 2007) had demonstrated that amyloid neurotoxicity in vitro is mediated by TNFSF10, and also that the latter is released by activated glial cells in response to inflammatory stimuli. Nevertheless, both TNFSF10 and its TNFRSF10B receptors are overexpressed after challenge with amyloid-β in human neuronal cells in vitro (Cantarella et al., 2003), supplying, at least in part, an explanation for increased cell death in amyloid-related disorders in experimental species and humans.

Interestingly, the increased expression of TNFSF10 observed in 3xTg-AD mice was significantly attenuated after treatment with a TNFSF10 neutralizing antibody.

There is a body of evidence that neutralization of TNFSF10 results in protection of intact neurons, which are susceptible to recruitment to apoptosis during neurodegeneration. For example, pretreatment with a TNFSF10 antiserum in mice undergoing spinal cord injury prevents progression of neuronal death and high expression of inflammatory molecules in the anterior horn cells and is associated with significant functional recovery (Cantarella et al., 2010). Also, incubation of human neuronal cells with anti-TNFSF10 antibodies prevents amyloid cytotoxicity to a large extent (Cantarella et al., 2003). Thus, not only are our observations corroborated by previous reports, but they also supply evidence that neutralization of TNFSF10 restrained cell death and inflammation during neurodegenerative processes occurs in a time-dependent fashion.

In fact, it is known that a major, yet unmet, objective of neuroprotective/anti-neurodegenerative treatments is to arrest or to slow down the fast progression of cognitive impairment (Patel and Holland, 2012). Interestingly, in parallel to the above ex vivo studies, behavioural experiments showed that the impairment in spatial learning, reference memory and recognition memory found in 3xTg-AD mice was prevented by an anti-TNFSF10 treatment.

When Caccamo et al. (2010) characterized the mTOR-related molecular mechanism of such cognitive decline in the 3xTg-AD mice, they reported concomitant appearance of a robust intraneuronal amyloid-β immunoreactivity, which increased thereafter in an age-related manner. In fact, it is noteworthy to remark on the blockade of mTOR induced by rapamycin (Wong, 2013), which is part of its immunosuppressive mechanism (Heinzl, 2000; Loewith and Hall, 2011). Consistently, the inhibition of the effect of the proinflammatory/proapoptotic cytokine TNFSF10 is well correlated with improved cognitive function. Such a result corroborates the concept that neuroinflammation is one prominent hallmark associated with amyloid-related damage (Broussard et al., 2012), and that cytokines of the TNF family, including TNFSF10, are substantial contributors to tissue damage during neurodegenerative processes (Huang et al., 2005). In fact, this is indirectly proven by elevated cytokine levels in the CSF of patients suffering from amyloid-related dementia (Genç et al., 2009).

Increased expression of inflammatory parameters was also found in the hippocampus of 3xTg-AD mice. Expression of IL1B (Shaftel et al., 2007), PTGS2/COX2 (Willard et al., 2000) and NOS2/NO (Zhou et al., 2013) is significantly augmented in injured neurons in many models of neuroinflammatory disorders. Consistent with our results, TNFSF10 immunoreactivity has been described in the vicinity of amyloid plaques of post-mortem human brain (Uberti et al., 2004). Thus it seems that TNFSF10 might induce cell death not only through interaction with its death receptor TNFRSF10B, but also by acting in a synergistic fashion with other cytokines.

In fact, it is a common notion that, for example, TNF released by activated macrophages, induces, in turn, the release of other inflammatory/apoptotic cytokines, such as IL1B (Claussen et al., 2008), which then act synergically. Such a consolidated principle supports our observation that shutting off release of TNFSF10, which triggers the cytokine cascade in the brain, would substantially restrain release of synergistic cytokines, such as IL1B, and thus, expression of inflammatory mediators, such as PTGS2 and NOS2, which sustain eventual progression of tissue damage.

The number of glial cells in the brain of 3xTg-AD mice was significantly increased, whereas it was diminished by treatment with an anti-TNFSF10 antibody, consistent with
decreased expression of the specific glial marker GFAP shown by protein and immunocytochemical analysis.

These data correlate with the movement of the inflammatory parameters discussed above, as it is known that glia activated by inflammatory stimuli release substantial amounts of inflammatory mediators and, among these, cytokines of the TNF family (He et al., 2002), including TNFSF10 (Cantarella et al., 2007). Gliosis is, in fact, a common feature of neurodegenerative disorders generally associated with inflammation (Kamphuis et al., 2014), and it seems a direct function of activation of glial cells (Eikelenboom et al., 2006).

In line with this, growing evidence substantiates the hypothesis that glia activation is relevant to Alzheimer’s disease pathogenesis (Querfurth and La Ferla, 2010). For example, astrocytes, which seem relevant to Alzheimer’s disease (Avila-Munoz and Arias, 2014), are constantly present in the brain of patients with Alzheimer’s disease (Kraft et al., 2013), and amyloid-β accumulates (Veeraraghavalu et al., 2014) and can induce morphological changes (Nagele et al., 2003) in these cells. Similarly, astrocytes, along with microglia, localize in the vicinity of fibrillary plaques (Wyss-Coray and Mucke, 2002), and chronically activated microglia release damaging cytokines such as IL1B, TNF and others (Akiyama et al., 2000).

Finally, immunofluorescence data demonstrate that the anti-TNFSF10 antibody injected intraperitoneally to both Alzheimer’s disease and wild-type mice at an early age, substantially localizes in the brains, thus confirming that IgG2α are able to cross the mouse blood–brain barrier (Banks, 2010).

In conclusion, for the first time, we demonstrate that neutralization of TNFSF10 results in dramatic functional recovery in models of severely impaired cognitive behaviour, such as 3xTg-AD. Such improvement is paralleled by reduced inflammatory mediators in the tissues. The effects of TNFSF10 imply activation of inflammatory mediators possibly involved and/or caused by associated gliosis, which increase their expression, thus exerting their detrimental effects locally in specific areas of the brain of these animals relatedly with development of amyloid plaques. When the expression of TNFSF10, its death receptors and related inflammatory mediators expression is reduced by a neutralizing treatment, the progression of such spontaneous cognitive impairment in 3xTg-AD significantly slows down with great beneficial effects on memory performance.

In conclusion, it seems plausible to hypothesize that TNFSF10 is a pivotal mediator of neuronal damage consequent to amyloid-related neuroinflammation. In fact, in our model, immunoneutralization of TNFSF10 implies a potent attenuating effect upon molecular mechanisms sustaining amyloid accumulation, paralleled by dramatic improvement of cognitive parameters in vivo. Targeting the TNFSF10 machinery with immunopharmacological modalities could be envisioned as a potential beneficial treatment for amyloid-related neurodegenerative disorders.

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