TRAIL-R Deficient Mice Are Protected From Neurotoxic Effects of Amyloid-β

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Abstract

TRAIL, a member of TNF superfamily, is a potent inducer of neuronal death. Neurotoxic effects of TRAIL appear mediated by its death receptor TRAIL-R2/DR5. To assess the role of TRAIL/TRAIL-R2 pathway in AD-related neurodegeneration, we studied the impact of the treatment with amyloid-β (Aβ) upon cell viability and inflammation in TRAIL-R-deficient mice (TRAIL-$R^{-/-}$). Here, we demonstrate that the lack of TRAIL-R2 protects from death cultured TRAIL-$R^{-/-}$ mouse embryonic hippocampal cells undergone treatment with either Aβ1-42 or TRAIL. Consistently, stereotaxic injection of Aβ1-42 resulted in blunted caspase activation, as well as in reduction of JNK phosphorylation and increased AKT phosphorylation in TRAIL-$R^{-/-}$ mice. Moreover, the lack of TRAIL-R2 was associated with blunted constitutive p53 expression in mice undergone Aβ1-42 treatment, as well as in decrease of phosphorylated forms of tau and GSK3β protein. Likewise, TRAIL-R2 appears essential to both TRAIL-Aβ mediated neurotoxicity and inflammation. Indeed, hippocampi of TRAIL-$R^{-/-}$ mice, challenged with Aβ1-42, showed a scanty expression of microglial (Iba-1) and astrocytic (GFAP) markers along with attenuated levels of IL-1β, TNF-a, iNOS and COX2. In conclusion, the bulk of these results demonstrate that the constitutive lack of TRAIL-R2 is associated with a substantial reduction of noxious effects of Aβ1-42, providing further evidence on the prominent role played by TRAIL in course of Aβ-related neurodegeneration and confirming that the TRAIL system represents a potential target for innovative AD therapy.

Introduction

Alzheimer's disease (AD) is the most common form of age-related dementia, characterized by an insidious onset of progressive cerebral atrophy and cognitive decline [1]. Distinctive neuropathological hallmarks of AD are represented by extracellular senile plaques of the amyloid-β (Aβ) peptide and by intracellular neurofibrillary tangles generated by hyperphosphorylated forms of the microtubule-binding protein tau [2]. Over the years, it has been demonstrated that many different factors such as genetics, free radicals, oxidative stress, mitochondrial dysfunction, or glutamate excitotoxicity may contribute to the pathogenesis and the progression of AD [3–6]. In addition, growing evidence suggests that AD pathogenesis is not restricted to the neuronal cell component, but it is significantly participated by an altered immune response [7, 8].

In fact, neuronal damage, along with Aβ deposition, triggers activation of microglial and astrocytic cells also through the release of cytokines belonging to the Tumor Necrosis Factor (TNF) superfamily [9, 10]. Among these, TNF-Related Apoptosis Inducing Ligand (TRAIL) is a potent proapoptotic molecule involved in either peripheral and central inflammatory processes [11, 12]. TRAIL, released by activated glia [13], CNS-infiltrating macrophages and damaged neurons, causes apoptosis in specific populations of cells in the CNS in course of neurodegenerative processes [14, 15], including those related to ischemia [16, 17], trauma [18], and Aβ accumulation [11, 19]. TRAIL exerts its biological effects through a complex ligand–receptor system encompassing five cognate receptors [20]. Cytotoxic effects of TRAIL are mediated by two death domain receptors, TRAIL-R1/DR4 and TRAIL-R2/DR5 [21, 22]. Interestingly, mice, unlike

humans, harbor only one of these two, mTRAIL-R2/mDR5, which shows 79% sequence homology with human DR5 [23]. Whereas TRAIL is not detectable in the healthy human brain, and its death receptors are scarcely expressed [24] its expression is abundant in the AD brain [25], and, consistently, death receptors expression is in turn proportionally increased [11]. In vitro data [26] showing that the blockade of the TRAIL receptor DR5, results in prevention Aβ-induced neurotoxicity, suggest that DR5 is a key mediator of the TRAIL apoptotic pathway. More recently, we have shown that immunoneutralization of TRAIL preserves cognitive behavior, along with reduction of Aβ deposits and blunted expression of inflammatory molecules in the mouse brain [11].

In neuronal cell lines DR5 expression is promoted by the tumor suppressor gene p53 [26], representing one main target gene, upregulated in response to DNA damage in different organs [27]. In addition, not only p53 directly regulates the expression of DR5, but also that of DR4, TRAIL-R3/DcR1 and TRAIL-R4/DcR2 [28–31].

Based upon this evidence, it appeared plausible to hypothesize a role of DR5 as a pivotal mediator of neuronal damage consequent to amyloid-related neuroinflammation. To address this hypothesis, the neurotoxic effects of Aβ have been assessed in a TRAIL-R deficient mouse (TRAIL-$R^{-/-}$) [32], a strain which develops normally and with an intact immune system, without defects in thymic negative selection [33].

Thus, we verified this hypothesis both in in vitro studies, using primary cultures of hippocampal cells originated from TRAIL-$R^{-/-}$ mice embryos, and in *in vivo* studies, wherein TRAIL-$R^{-/-}$ mice were stereotactic injected in the dentate gyrus of the hippocampus with neurotoxic oligomers of Aβ1-42 with the aim to generate an AD phenotype.

Materials And Methods

Animals

TRAIL-$R^{-/-}$ animals were kindly provided by Prof. H. Walczak, Dept of Cancer Biology, CRUK-UCL Cancer Ctr., UCL Cancer Inst., London, United Kingdom. TRAIL-$R^{-/-}$ animals and wild-type (WT) littermates in the B6 background 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 approved by the Italian Ministry of Health (authorization n.86/2015 PR) and conducted following the European Community directive guidelines for the use of animals in laboratory (2010/63/EU) and the Italian law (D.Lgs. 26/2014).

Preparation of Aβ1-42 Oligomers

Aβ1–42 oligomers were generated as the previously described method [34]. Briefly, the Aβ1–42 lyophilized peptide (Sigma-Aldrich, St. Louis, MO, USA) was initially dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Sigma-Aldrich) to a final concentration of 1 mM and incubated at room temperature for 2 h. The peptide solution was aliquoted and dried in the fume hood. Traces of HFIP was removed under vacuum in a SpeedVac centrifuge (800 g, RT), and the thin clear peptide film was stored over desiccant at −80°C. For aggregation, the aliquoted peptide film was dissolved in dimethyl sulfoxide (DMSO) to 5 mM.

The peptide in DMSO was diluted directly into sterile phosphate buffered saline (PBS, 1×) at 100 μM and incubated at 4°C for 12 h to make the oligomeric form of Aβ1–42. Following incubation, Aβ1-42 samples were immediately used for the cell treatment or aliquoted and stored at –20°C until their use.

Experimental Groups and Drug Administration

Twenty TRAIL-$R^{-/-}$ and 20 wild-type C57BL/6J male mice were enrolled at nine months of age and four study groups were used: (i) Wild-type plus vehicle; (ii) Wild-type plus Aβ1-42; (iii) TRAIL-$R^{-/-}$ plus vehicle; and (iv) TRAIL-$R^{-/-}$ plus Aβ1-42.

As previously described [35], mice were positioned on a stereotaxic frame and a Hamilton syringe with a 29 G needle was implanted into the dentate gyrus of the hippocampus using the following stereotaxic coordinates from the bregma: AP, -2.00 mm; ML, ±1.3 mm; DV, -2.2 mm. Animals were treated with oligomeric Aβ1-42 or vehicle and sacrificed after 2 weeks.

Primary Cultures of Mouse Hippocampal Neurons

Sixteen embryonic day mice were obtained from surgically sacrificed pregnant mouse and the hippocampus was sepa-rated under surgical stereomicroscope. Separated tissues were isolated and dissociated by manual dispersion with a fire-polished Pasteur pipette. Cells were plated at a density of 1.5 × 10^5 cells/cm^2. The cells were plated in Neurobasal medium (Invitrogen Corporation, Waltham, MA, USA) supplemented with 2% B27 (Invitrogen Corporation), 0.5 mM L-glutamine, and 50 U/ml penicillin/streptomycin (Invitrogen Corporation). Three days after plating, 50% of the medium was changed with fresh medium and subsequently 50% of the medium was changed twice a week, until 11 days in vitro. To inhibit glial cell outgrowth, cytosine arabinoside (1 μM) was added at the moment of media change.

Cell Viability Assay

Cell viability was determined by 3-[4,5 dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide assay. At the end of each treatment, cell viability was measured by the reduction of 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide solution (0.5 mg/mL). The solution was removed after 3 h of incubation at 37°C and dimethylsulfoxide was added to ob-tain cell lysis and solubilization of blue formazan crystals resulting from MTT reduction by viable cells' mitochondrial activity. Optical density of the blue formazan was measured at 570 nm with a VarioskanTM Flash Multimode Reader (Thermo Fisher Scientific, Waltham, MA, USA).

Free-Floating Fluorescence Immunohistochemistry

Mice were deeply anesthetized by i.p. injection of Zoletil 100 (40 mg/kg) (Virbac S.r.l., Milan, Italy) and perfused trancardially with 4% paraformaldehyde (PFA) solution in 0.1 M phosphate buffer (PBS; pH 7.4). The brains were removed, post-fixed overnight in the same 4% PFA and then transferred into a 30% sucrose in PBS as cryo-protective solution at 4°C for 2–3 days. Serial 25 μm frozen hippocampal

sections of the brains were cut and subjected to immunohistochemical assay. Briefly, free-floating sections were washed three times in PBS and then blocked at room temperature for 1 h in 5% normal goat serum (NGS) in PBS. They were then incubated overnight at 4°C with the following antibodies: a mouse monoclonal anti-Iba1 (1:200, Abcam, Cambridge, United Kingdom) as microglial marker and a mouse anti-GFAP antibody (Cell Signaling Technology, Inc., Danvers, MA, United States). For fluorescence visualization, after washing in PBS three times for 5 minutes each, sections were incubated in the dark for 1h at room temperature with the corresponding fluorescent-labelled secondary antibodies: Alexa Fluor 546 donkey anti-mouse IgG (1:500, Thermo Fisher Scientific) and Alexa Fluor 488 goat anti-mouse IgG (1:500, Thermo Fisher Scientific). Finally, sections were washed in PBS three times for 5 minutes each and mounted on gelatinated slides. Digital images were captured with a Zeiss Observer.Z1 microscope equipped with the Apotome.2 acquisition system (Zeiss, Oberkochen, Germany).

Protein Extraction

Freshly isolated hippocampal tissues were lysed in a buffer containing 150mM NaCl, 50mM Tris–HCl (pH 7.5), 5mM EDTA, 1mM Na_3VO_4, 30mM sodium pyrophosphate, 50mM NaF, 1mM acid phenyl-methyl-sulphonyl-fluoride, 5 µg/ml aprotinin, 2 µg/ml leupeptin, 1 µg/ml pepstatin, 10% glycerol, and 0.2% Triton X-100. The homogenates were then centrifuged at 14000 rpm for 10 min at 4°C and supernatants were collected. Protein concentration of the supernatant was determined by the Bradford method [36].

Western Blot Analysis

Equal amounts of protein (50 µg) were separated by 8%-12% SDS-PAGE gels and transferred onto Hybond ECL nitrocellulose membranes (Amersham Life Science, Buckinghamshire, UK). The membranes were blocked with 5% non-fat dry milk in PBST for 1 hour at RT and were then probed overnight at 4°C on orbital shaker with the following appropriate primary antibodies: rabbit anti-DR5 (1:500; Abcam, Cambridge, United Kingdom), mouse anti-p53 (1:1000; Cell Signaling Technology, Inc., Danvers, MA, United States), rabbit anti-DcR1 (1:1000; Abcam), mouse anti-p-JNK (1:500; Santa Cruz Biotechnology Inc.), mouse anti-JNK (1:500; Santa Cruz Biotechnology), rabbit anti-p-AKT (1:1000; Cell Signaling Technology, Inc.), rabbit anti-AKT (1:1000; Cell Signaling Technology, Inc.), mouse anti-p-GSK3β (1:500; Becton Dickinson, Franklin Lakes, NJ, USA), mouse anti-GSK3β (1:500; Santa Cruz Biotechnology Inc.), mouse anti-p-TAU (1:250; Santa Cruz Biotechnology Inc.), rabbit anti-TAU (1:500; Santa Cruz Biotechnology Inc.), mouse anti-Iba-1 (1:500; Abcam), mouse anti-GFAP (1:500; Cell Signaling Technology), rabbit anti-NOS2 (1:500; Santa Cruz Biotechnology Inc.), mouse anti-COX2 (1:500; Santa Cruz Biotechnology Inc.), rabbit anti-IL-1β (1:250; Santa Cruz Biotechnology Inc.) and rabbit anti-TNF-α (Novus Biologicals, Centennial, CO, USA). Beta-tubulin (Santa Cruz Biotechnology Inc.) primary antibody was used as an internal control to validate the right amount of protein loaded in the gels. Then the membranes were washed with PBS-T and probed with the appropriate horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Milan, Italy) for 1 hour at room temperature in 5% non-fat dry milk. After washing with PBS-T, protein bands were visualized by enhanced chemiluminescence (Thermo Fisher Scientific) and scanned with the iBright FL1500 Imaging System (Thermo Fisher Scientific).

Densitometric analysis of band intensity was performed with the aid of ImageJ software (https://imagej.nih.gov/ij/).

Caspase Colorimetric Assay

Caspase activities were measured with the Caspase Colorimetric Substrate Set II Plus kit (BioVision Inc., Milpitas, CA, USA) as previously described [37]. In brief, protein extracts (100 µg) were added to a reaction buffer containing a p-nitroanilide-labelled specific caspase substrate and incubated for 2h at 37°C. Relative caspase activity was measured as optical density at 405 nm in a microplate reader (Bio-Rad Laboratories, Inc., Italy). Fold-increase in caspase activity was determined by comparing results with the level of the uninduced control.

Nitrite Assay

Nitric oxide production in the primary embryonic hippocampal neurons was assessed by the Griess method, as previously described [13]. Briefly, after 24 h treatments, 100µL aliquots of culture supernatants were incubated with 100 µL of Griess reagent (1% sulphanilamide, 0,1%N-(1-naphtil)ethyl-enediamine dihydrochloride and 5% of phosphoric acid) at room temperature for 20 min. Optical density at 540 nm was determined using a microplate reader (Bio-Rad Laboratories). The nitrite concentration was determined from a sodium nitrite standard curve.

Statistical Analysis

Data were analyzed by the one-way analysis of variance (ANOVA) test, followed by the Bonferroni post-hoc test. Data were represented as means ± standard error mean (SEM). Significance was set at a $p<0.05$. Graph design and statistical analyses were carried out with the dedicated software GraphPad Prism (La Jolla, CA, USA).

Results

Amyloid beta neurotoxicity is significantly attenuated in TRAIL-R$^{-/-}$ mouse primary neuronal cells

To better understand the role of TRAIL system in neurotoxic processes related to Aβ, in vitro cell viability experiments were performed on primary cultures of embryonic hippocampal cells derived from WT and TRAIL-R$^{-/-}$ mice. Cells were challenged for 48h with Aβ1-42 (1 µM), TRAIL (100 ng/ml), an anti-TRAIL antibody (1 µg/ml), or various combinations of these substances (Fig. 1). Aβ neurotoxicity, assessed in hippocampal cells from WT mice 48 hours after treatment, was significantly cultured attenuated in hippocampal cells from TRAIL-R$^{-/-}$ mice. Moreover, the treatment with an anti-TRAIL neutralizing antibody resulted in significant rescue of embryonic hippocampal cells of WT mice from the neurotoxic effect of TRAIL and Aβ1-42 (Fig. 1). Overall, hippocampal cells from TRAIL-R$^{-/-}$ mice showed a significantly higher

resistance to death induced by both Aβ1-42 and TRAIL, as compared to cells from TRAIL-R-proficient mice.

TRAIL-R2 is required for p53 to mediate Aβ-related neurotoxicity

To better understand the role of the TRAIL-R2 in Aβ mediated neurotoxicity, we generated an AD phenotype in TRAIL-$R^{-/-}$ mice, by performing stereotaxic injection of the neurotoxic oligomer Aβ1-42 into the dentate gyrus of the hippocampus. Since it is well known that the tumor suppressor gene p53 is involved in Aβ neurotoxicity [38–40] and that both TRAIL-R2 and DcR1 receptors are p53 target genes [29, 30], we investigated the expression of these proteins in hippocampi from WT and TRAIL-$R^{-/-}$ mice treated for two weeks with oligomeric Aβ1-42. Western blot analysis revealed that, while the expressions of p53 and TRAIL-R2 were significantly enhanced in WT mice treated with Aβ1-42, p53 expression was blunted in TRAIL-$R^{-/-}$ mice treated with Aβ1-42. DcR1 expression, was up-regulated in WT mice receiving Aβ1-42 treatment, whereas DcR1 was down-regulated in TRAIL-$R^{-/-}$ mice treated with Aβ1-42 (Fig. 2). These results suggest that TRAIL-R2 may represent a crucial element for p53 to mediate Aβ-related neurotoxicity.

TRAIL-$R^{-/-}$ mice show reduced caspase activity after challenge with Aβ1-42

Activation of the TRAIL-R2 is associated with the recruitment of caspase-8 and consequent activation of the caspase cascade leading to cell death [41]. We studied this pathway in cultured embryonic hippocampal cells derived from WT and TRAIL-$R^{-/-}$ mice (Figure 3A). Cells were incubated for 48 hr with either Aβ1-42 (1 μM), TRAIL (100 ng/ml), the pan-caspase inhibitor z-VAD-FMK (2 μM), or various combinations of these substances. While cell viability of TRAIL-$R^{-/-}$ embryonic hippocampal cells and then challenged with Aβ1-42 or TRAIL was not affected by the treatment with the caspase inhibitor z-VAD-FMK, cells from WT mice, pre-treated with z-VAD-FMK and treated with either Aβ1-42 or TRAIL, showed significant increase of viability compared to those treated with Aβ1-42 alone, or TRAIL, but in the absence of z-VAD-FMK. These results suggested that the lack of expression of the TRAIL-R2 and the subsequent inactivation of caspases lead to substantial protection from Aβ or TRAIL- induced neuronal death.

To confirm the role of TRAIL-R2 in mediating Aβ neurotoxicity, we also assessed the activity of caspase-3, -8 and -9 in lysates of hippocampi from WT and TRAIL-$R^{-/-}$ mice treated with oligomeric Aβ1-42. Hippocampi from TRAIL-$R^{-/-}$ mice treated with oligomeric Aβ1-42 showed decreased enzymatic activity of either caspase-8, as well as caspases -3 and -9, confirming data obtained with cultured hippocampal cells from TRAIL-$R^{-/-}$ (Fig. 3B). These findings corroborate the hypothesis that the lack of expression of the TRAIL-R receptor is associated with unability to activate caspases, leading to substantial protection from Aβ-induced neuronal death.

JNK and AKT kinases are inversely modulated in TRAIL-R$^{-/-}$ mice undergone Aβ1-42 treatment

Downstream activity of death receptors involves a protein family named stress-cytokines-induced kinases, which include c-Jun N-terminal kinase (JNK), whose phosphorylation requires the cleavage of caspase-3, -8 and -9 [37, 42, 43]. Therefore, the involvement of this kinase was investigated in our model. Indeed, western blot analysis showed that JNK phosphorylation was significantly increased in WT mice undergone Aβ treatment, compared to TRAIL-R$^{-/-}$ mice treated with Aβ, which, on the other hand, essentially displayed just basal levels of JNK phosphorylation (Fig. 4A). The inhibition of JNK phosphorylation is in turn associated with activation of the AKT pathway, a central node in cell signaling downstream of growth factors, cytokines, and other cellular stimuli [44]. Western blot analysis confirmed that phosphorylation of AKT protein was indeed inversely correlated with JNK phosphorylation. In fact, whereas AKT phosphorylation was significantly blunted in WT mice undergone to Aβ, it was significantly increased in TRAIL-R$^{-/-}$ mice treated with Aβ (Fig. 4B).

Aβ1-42 dependent GSK3β activation and Tau phosphorylation are naturally attenuated in TRAIL-R$^{-/-}$ mice

Glycogen synthase kinase-3β (GSK3β), an ubiquitously expressed serine/threonine kinase, plays a key role in the pathogenesis of AD, influencing Tau phosphorylation, amyloid-β production, neurogenesis and synaptic function, and, finally memory [45]. In particular, GSK3β phosphorylation/activation is consequent to a challenge of neurons with Aβ or TRAIL [21]. For this reason, we checked whether the lack of TRAIL-R2 could be of help to better understand the role of TRAIL signaling pathway in this process. We in fact demonstrated that, while TRAIL-R2 did not affect the constitutive expression of GSK3β, the expression of its phosphorylated form was increased in WT mice undergone Aβ treatment and, on the other hand, it was significantly decreased in TRAIL-R$^{-/-}$ mice treated with Aβ (Fig. 5A).

Hyperphosphorylation of Tau protein is a process downstream the GSK3β phosphorylation/activation [45] and is regarded as a major step in either the Aβ- or TRAIL-induced neuronal death [37]. Therefore, in order to assess whether the lack of TRAIL-R2 could impact upon Tau phosphorylation rate, the expression of both Tau and p-Tau was analyzed by western blot on hippocampal lysates from the same groups of animals mentioned above. TRAIL-R2 affected tau phosphorylation significantly, as it was highly expressed in WT mice treated with Aβ1-42, while its expression was significantly attenuated in TRAIL-R$^{-/-}$ mice treated with Aβ1-42 (Fig. 5B).

Glial response is deficitary in TRAIL-R$^{-/-}$ mice treated with Aβ1- 42

In order to investigate the contribution of the TRAIL system to neuroinflammation with regard to microglia and astrocyte activation, the expression of microglia and astrocyte markers, respectively, Iba-1 and GFAP,

were studied in the hippocampi from WT and TRAIL-R$^{-/-}$ mice treated for 2 weeks with oligomeric Aβ1-42, administered stereotactically.

Immunofluorescence experiments revealed widespread glia activation in the brain of WT mice treated with Aβ1-42, as represented by high expression of astrocytic and microglial markers, respectively GFAP and Iba-1. Interestingly, the expression of both markers was significantly reduced in the hippocampus of TRAIL-R$^{-/-}$ mice treated with oligomeric Aβ1-42 (Fig. 6A). These data were confirmed by protein expression levels of GFAP and Iba-1, as assessed by western blot analysis. (Fig. 6B).

These results demonstrate that the TRAIL system may play a pivotal role in glia activation occurring in course of neuroinflammation triggered by noxious stimuli such as oligomeric Aβ1-42.

Inflammatory molecules expression is blunted in TRAIL-R$^{-/-}$ mice receiving Aβ1-42

As gliosis is a common pathological feature of neurodegenerative processes and it is intimately associated with neuroinflammation, we verified whether the TRAIL system could also modulate glial expression and release of pro-inflammatory mediators. Thus, either NOS2, COX2, IL-1β and TNF-α proteins were analyzed by means of western blot in respective hippocampal lysates.

Results showed that, while NOS2, COX2, IL-1β and TNF-α were expressed substantially in WT mice treated with Aβ1-42, their expression was significantly reduced in WT mice treated with vehicle and in TRAIL-R-/- mice, suggesting that the TRAIL pathway provides a decisive contribution to neuroinflammation-related neurodegeneration and is associated with a substantial decline of the expression of noxious molecules induced by Aβ1-42 (Fig. 7).

Nitrite levels are significantly attenuated in the media from embryonic hippocampal cell cultures from TRAIL-R$^{-/-}$ mice treated with Aβ1-42

Nitric oxide reactive derivatives have been implicated as non-specific inflammatory mediators of neuronal death in several neurodegenerative and neuroinflammatory conditions including AD [46]. In particular, reactive nitrogen oxide species are reported to create some sort of closed cycle where they trigger Aβ deposition, which in turn might activate immune cells [47].

With the purpose of validating, from a functional point of view, the reduced expression levels of NOS2 previously achieved by Western blot analysis in TRAIL-R$^{-/-}$ mice, we investigated nitrite release in the media of embryonic hippocampal cells derived from WT and TRAIL-R$^{-/-}$ mice challenged for 24h with LPS (10 μg/ml) Aβ1-42 (1 μM), TRAIL (100 ng/ml), anti-TRAIL antibody (1 μg/ml), or various combinations of these compounds. Results revealed that the constitutive lack of TRAIL-R2 is associated with a significative reduction of nitrite levels in the various experimental conditions studied (Fig. 8).

Discussion

Here, we studied the neurotoxic effects of Aβ in TRAIL-R$^{-/-}$ mice [33] showing that, indeed, the TRAIL/TRAIL-R system could play a critical role in neuronal damage consequent to amyloid-related neuroinflammation.

Specifically, the role of TRAIL system in neurotoxic processes related to Aβ has been better defined in in-vitro cell via-bility experiments performed on primary embryonic hippocampal cells derived from WT and TRAIL-R$^{-/-}$ mice, which showed a significantly higher resistance to death induced by both Aβ1-42 or TRAIL. This is in line with previous findings showing that neutralization of TRAIL death pathway protects human neurons from Aβ toxicity [19].

It is well documented that the tumor suppressor gene p53 is a pleiotropic transcription factor that plays a crucial role in determining cell fate under certain conditions, including excitotoxicity, ischemic injury, ionizing radiation, and oxidative stress. p53 is constitutively present in many cell types including neurons and is upregulated and activated via phosphor-ylation following these various insults resulting in transactivation of different target genes which control both the pro-cesses of cell survival and death [48]. It is noteworthy that both TRAIL-R2 and DcR1 receptors are p53 target genes [29] and, while is clear that TRAIL-R2 upregulation is depending upon p53 activation [49], the requirement of TRAIL-R2 during p53-mediated apoptosis remains still unclear. However, more recently nuclear TRAIL-R2 has been shown to act as a negative regulator of p53, suggesting that it may heavily impact, for example, on tumor growth [50]. Despite these findings, we found that the p53 expression was blunted in TRAIL-R$^{-/-}$ mice treated with Aβ1-42, suggesting that TRAIL-R2 contributes, in turn, to p53 mediation of Aβ-induced neurotoxicity. Consistently, p53 is downregulated in the white matter of DR5 null mice, a strain protected from radiation induced cell death, supporting the role of p53-depending TRAIL-R2-related cell death in the central nervous system [51].

The downstream signal of TRAIL-R2 involves the engagement of the caspase cascade that induces apoptosis [41]. As a matter of fact, in our experiments the lack of TRAIL-R2 expression correlates with a reduced activity of the caspase cascade, leading to effective protection from Aβ-induced neuronal death. Moreover, it is well documented that Aβ is able to induce the expression of TRAIL both in vitro [19] and in vivo [11] and that both TRAIL and Aβ may affect different pathways, including the stress-cytokines-induced kinase, such as JNK [37, 52], as well as the serine/threonine kinase AKT, known to regulate numerous processes, including cell survival, growth, and apoptosis [53]. Following treatment with Aβ, we found lower levels of JNK phosphorylation in the hippocampi of TRAIL-R$^{-/-}$ mice and, on the other hand, in-creased levels of AKT phosphorylation. These results corroborate the concept that the TRAIL-R2 may indeed represent an essential element to trigger and fuel the cascade of events related to neurodegeneration in this model. As a proof, the lack of TRAIL-R2 is associated with failure in initiating the complex, multifactorial intracellular signaling machinery set into motion by Aβ-depending TRAIL neurotoxicity.

In the latter, GSK3β represents one of the main enzymes responsible for hyperphosphorylation of the tau protein, which is a typical hallmark of AD-related neuroinflammation and apoptosis [45]. Either Aβ [52] or TRAIL [37], induce either phosphorylation of JNK and dephosphorylation of AKT, events both connected with increased GSK3β phosphorylation, functional, in turn, to Tau hyperphosphorylation [37]. Consistently, we found that, in front of the significant Aβ-dependent increase of GSK3β phosphorylation and consequent Tau hyperphosphorylation, occurring in the hippocampus of wild type animals, mice lacking the TRAIL-R2 were not performing these features. These data endorse the engagement of TRAIL-R2 as a critical event to the neurodegenerative process driven by Aβ via Tau hyperphosphorylation.

Moreover, TRAIL is known to induce gliosis, another typical feature of AD brain pathology [54]. In our hands, the number of GFAP and Iba-1 positive cells was negligible in the brain of TRAIL-R-/- animals challenged with Aβ oligomers, supporting the hypothesis that the TRAIL system plays a pivotal role in sustaining activation of glia to fuel the neuroinflammatory machinery. Such increased number of active microglia and astrocytes, is consistent with abundant expression and release of inflammatory mediators, such as NOS2, COX2, IL-1β and TNF-α [55, 56]. It is noteworthy how a TRAIL-neutralizing treatment results in significant decrease of both cellular and soluble factors contributing to brain inflammation [11], suggesting that TRAIL is a coordinating molecule in the inflamed brain, as demonstrated, for example, by its capability to control capabilities on peripheral immunocytes trafficking in the brain of 3xTg-AD mice [57]. Results obtained in our experiments conducted on TRAIL-$R^{-/-}$ mice show that Aβ is not able to induce gliosis and increased expression of inflammatory molecules. Thus, our data finally confirm and corroborate the concept that the TRAIL system is essential to set into motion and eventually drive sustained inflammatory/immune response in the AD brain.

Conclusions

Overall, our experiments demonstrated a pivotal role of the TRAIL-R2 in the pathogenesis of Alzheimer's disease in a murine model. We showed that the lack of the TRAIL-R2 is associated with substantial restraint of noxious effects of Aβ, providing genetically assessed evidence that the TRAIL/TRAIL-R system activated during neuroinflammatory processes, is responsible for Aβ-induced neurotoxicity. On the basis of these results, the TRAIL system may be envisioned as a potential candidate target for effective therapeutic intervention in AD.

Declarations

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Author Contributions

R.B. and G.C. designed the research; G.D. and C.B. performed the research; G.D.B. and M.F.S. conducted the ethical experiments on animals, C.M.B. and R.D. analyzed data; G.D.B., A.M., R.B. and G.C. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability

The data presented in this study are available from the corresponding author on reasonable request.

Ethics Approval

All experiments were approved by the Italian Ministry of Health (authorization n.86/2015 PR) and conducted following the European Community directive guidelines for the use of animals in laboratory (2010/63/EU) and the Italian law (D.Lgs. 26/2014).

Consent to Partecipate

Not applicable.

Consent for Pubblication

Not applicable.

Conflicts of Interest

The authors declare no conflict of interest.

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Figures

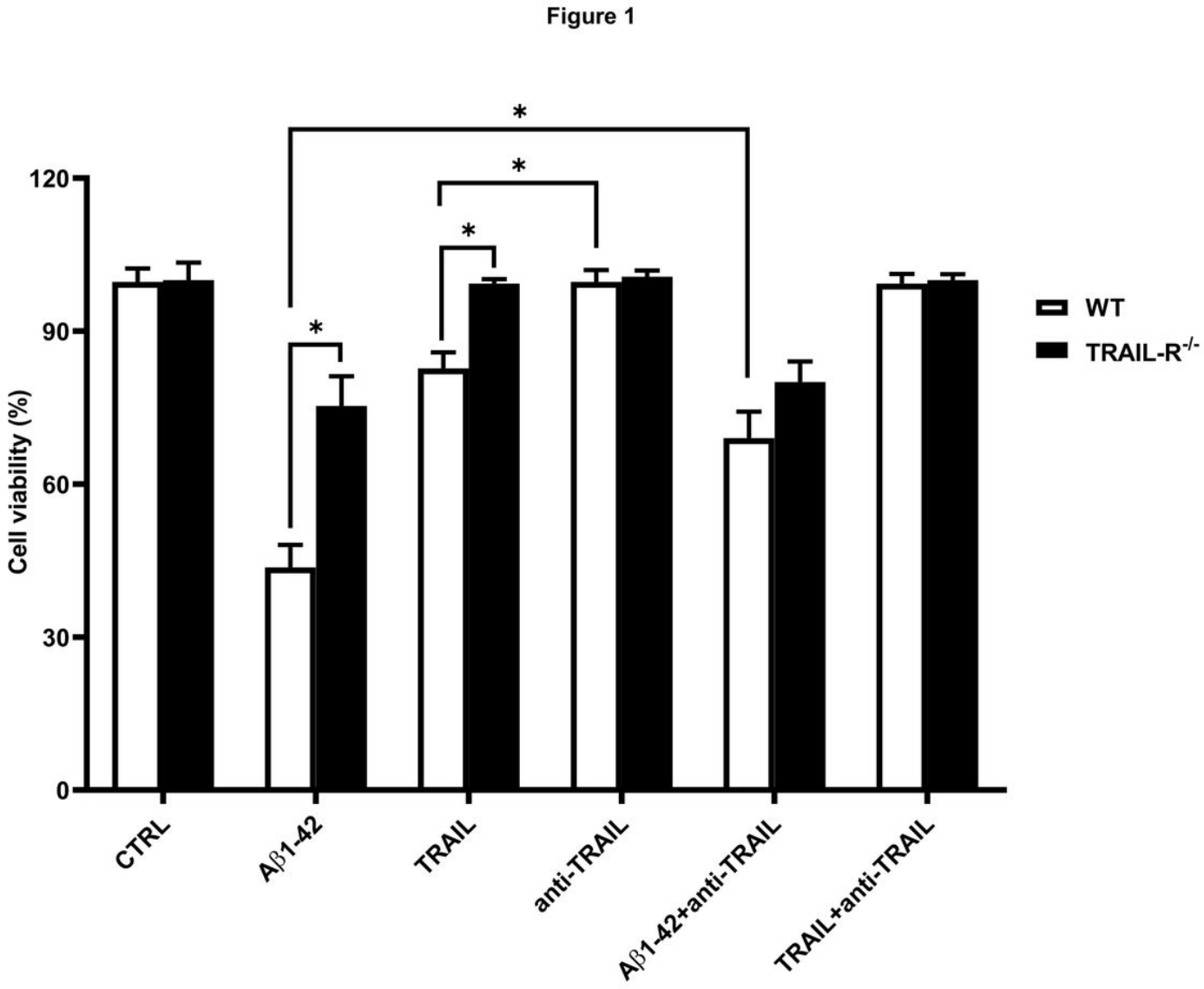

Figure 1

Amyloid beta neurotoxicity is attenuated in TRAIL-R-/- mouse primary hippocampal cells. Cell viability of primary embryonic hippocampal neurons from WT and TRAIL-R-/- mice, following 48h treatment with Aβ1-42 (1 μM), TRAIL (100 ng/ml), anti-TRAIL antibody (1 μg/ml), or various combinations of the compounds. Vertical bars are means ± standard error mean (SEM). One-way ANOVA and the Bonferroni post-hoc test were used for statistical analysis. *p< 0.05.

Figure 2

TRAIL-R2 is essential for p53 to mediate Aβ-related neurotoxicity. (A) Western blot for TRAIL-R2, p53 and DcR1 protein expression in the hippocampus of WT and TRAIL-R-/- mice following stereotaxic infusion of oligomeric Aβ1-42 or vehicle. (B) Densitometric analysis of TRAIL-R2, (C) p53 and (D) DcR1 western blots. Data are expressed as means ± SEM. One-way ANOVA and the Bonferroni post-hoc test were used to determine statistical significance. *p <0.05. N=5 animals for each group.

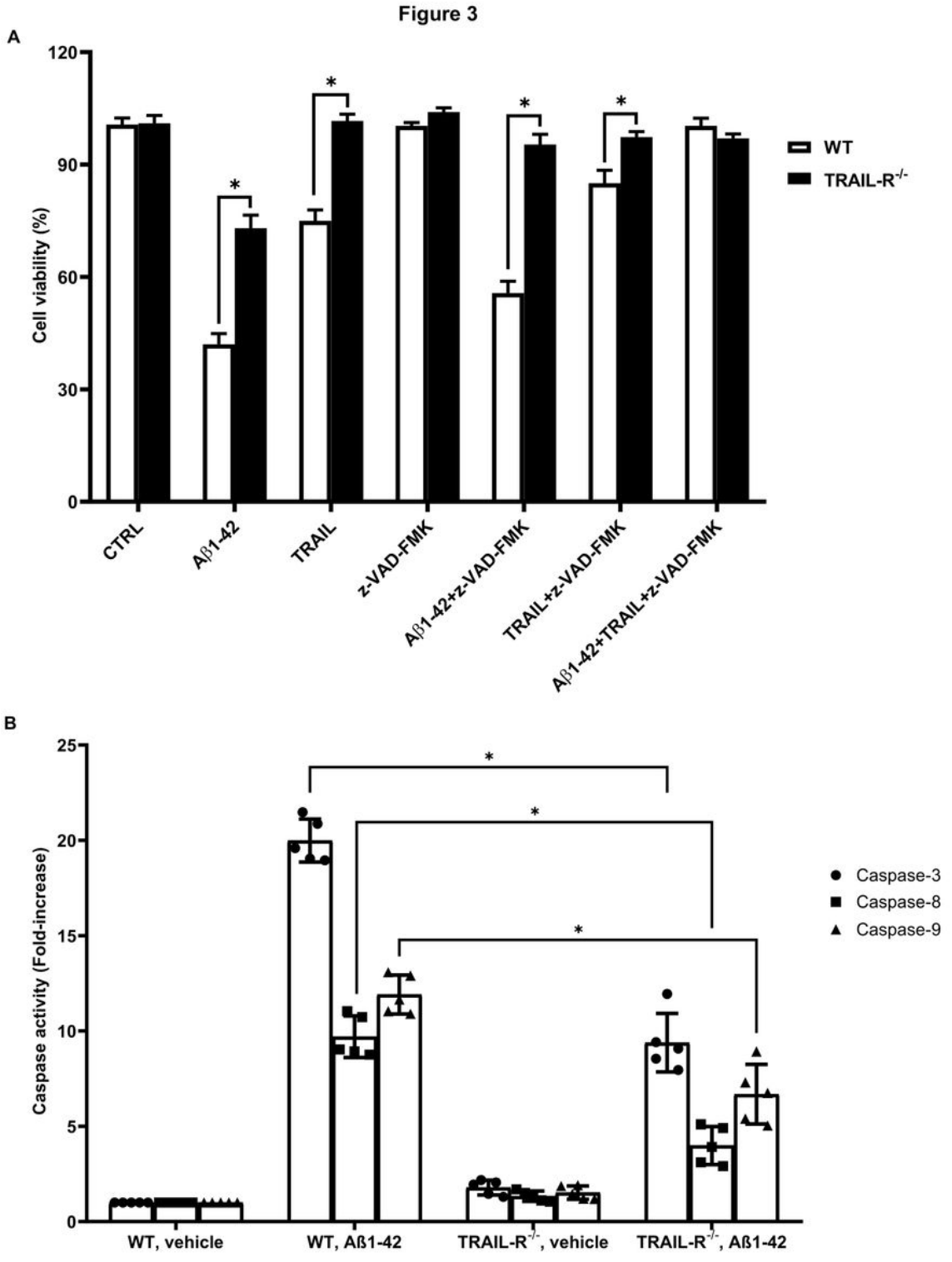

Figure 3

Reduced caspase activity in TRAIL-R-/- mice challenged with Aβ1-42. (A) Embryonic hippocampal cell viability following 48h treatment with Aβ1-42 (1 μM), TRAIL (100 ng/ml), z-VAD-FMK (2 μM) or various combinations of the compounds. Data are expressed as means ± SEM. One-way ANOVA and the Bonferroni post-hoc test were used to determine statistical significance. *p <0.05. (B) Caspase-3, -8 and -9 activity in WT and TRAIL-R-/- mice following stereotaxic infusion of oligomeric Aβ1-42 or vehicle. Data

are expressed as means ± SEM. One-way ANOVA and the Bonferroni post-hoc test were used to determine statistical significance. *p <0.05. N=5 animals for each group.

Figure 4

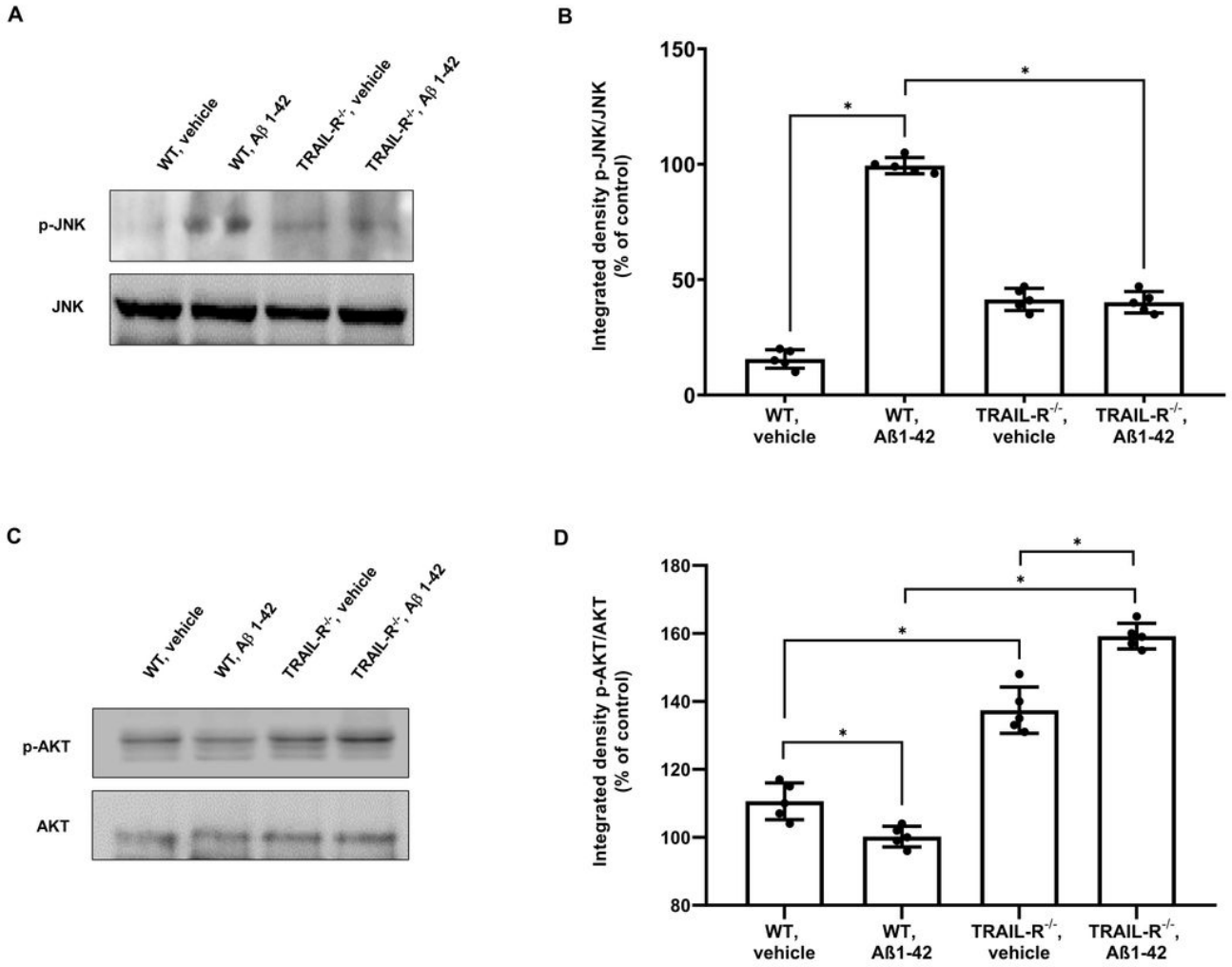

Figure 4

Inverse modulation of JNK and AKT kinases in TRAIL-R-/- mice undergone Aβ1-42 treatment. (A) Western blot analysis of p-JNK and (C) p-AKT in WT and TRAIL-R-/- mice following stereotaxic infusion of oligomeric Aβ1-42 or vehicle. (B) and (D) are respective densitometric analysis of the western blots. Data are expressed as means ± SEM. One-way ANOVA and the Bonferroni post-hoc test were used to determine statistical significance. *p <0.05. N=5 animals for each group.

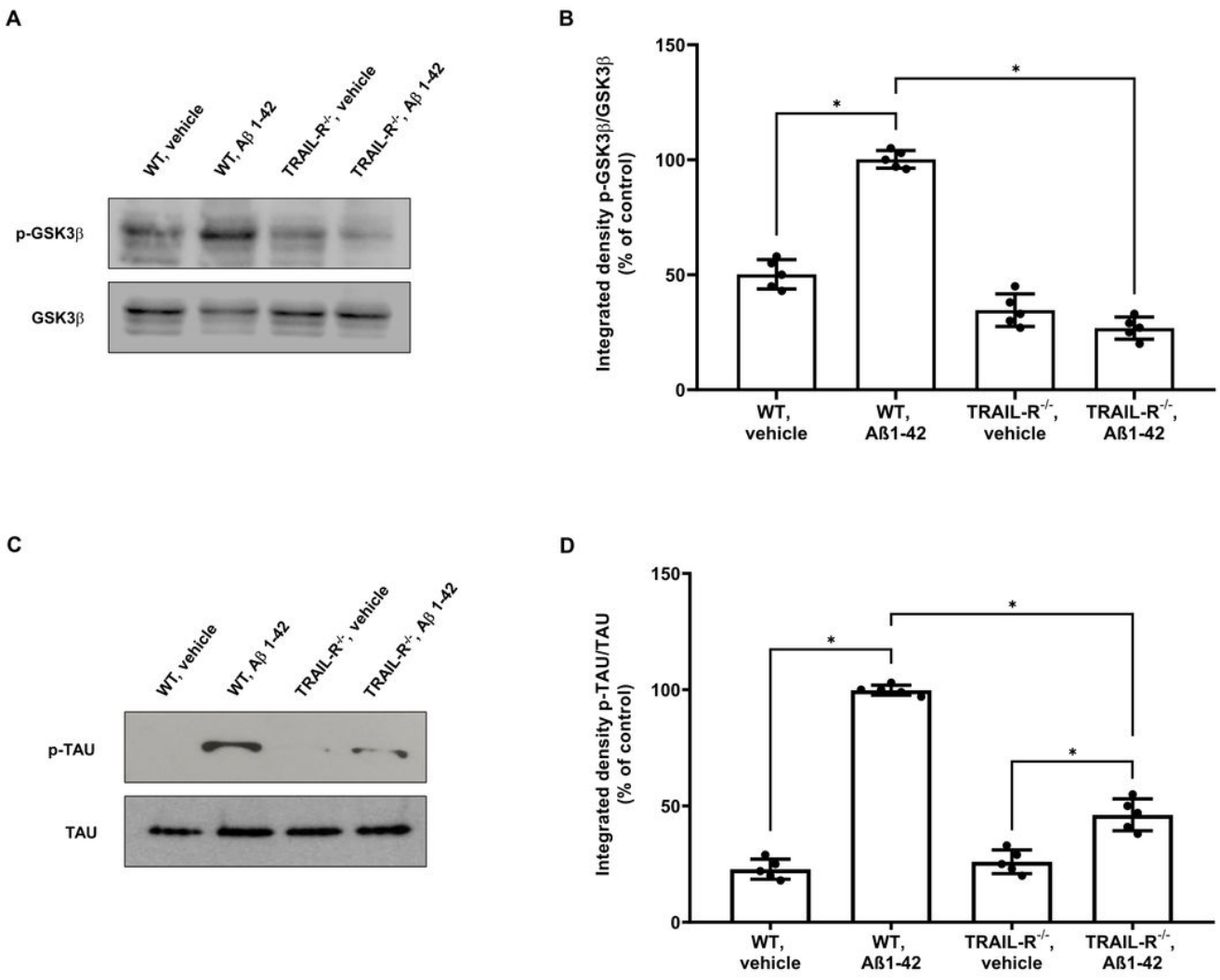

Figure 5

Phosphorylation of GSK3β and Tau is attenuated in TRAIL-R-/- mice treated with oligomeric Aβ1-42. Western blot analysis of (A) p-JNK and (C) p-AKT in WT and TRAIL-R-/- mice following stereotaxic infusion of oligomeric Aβ1-42 or vehicle. (B) and (D) are respective densitometric analysis of the western blots. Data are expressed as means ± SEM. One-way ANOVA and the Bonferroni post-hoc test were used to determine statistical significance. *p <0.05. N=5 animals for each group.

Figure 6

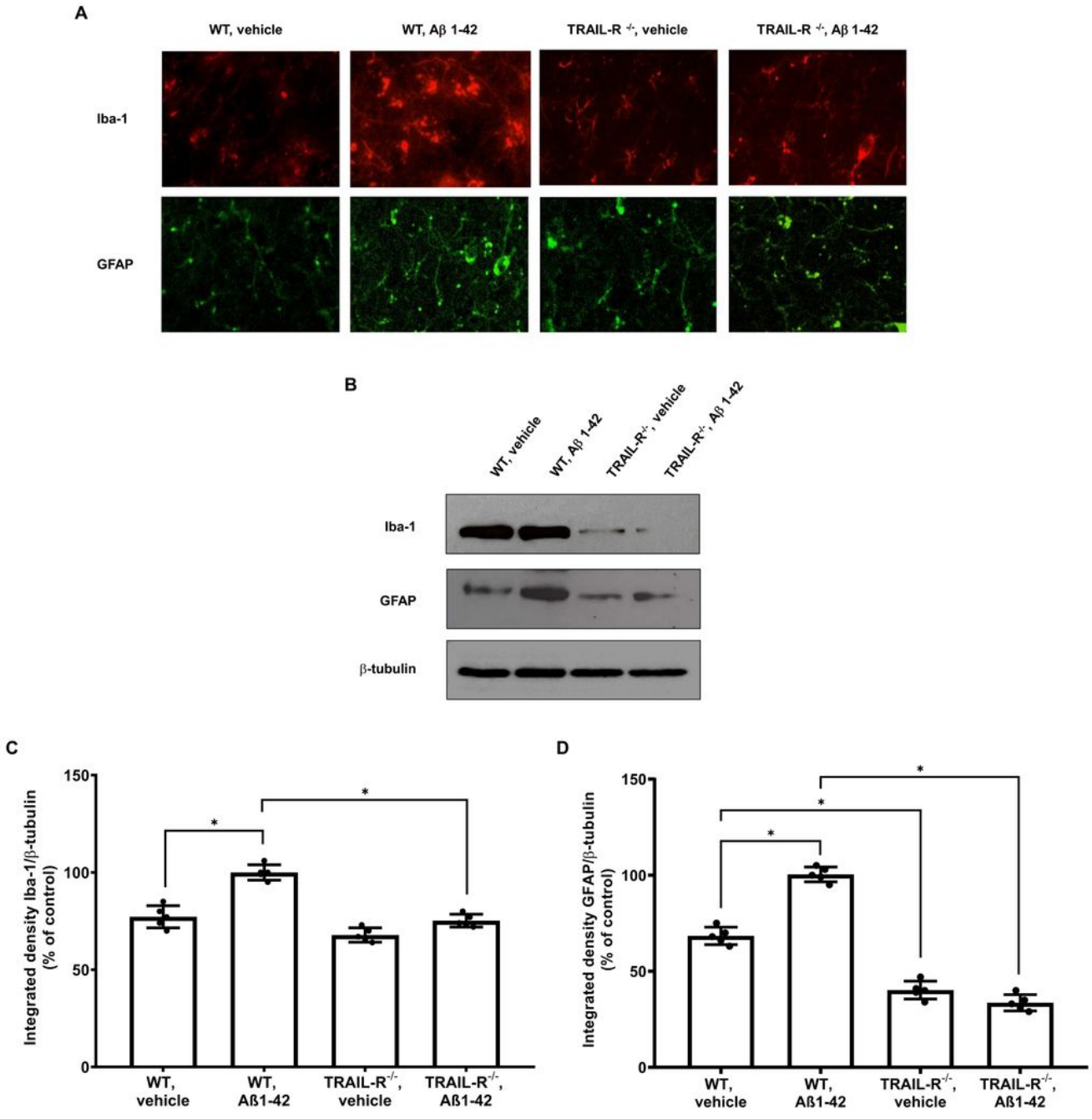

Figure 6

GFAP and Iba-1 are significantly reduced in the hippocampus of TRAIL-R-/- mice treated with oligomeric Aβ1-42. (A) Representative images of the fluorescent immunohistochemical detection of Iba-1 and GFAP expression in WT and TRAIL-R-/- mice following stereotaxic infusion of oligomeric Aβ1-42 or vehicle. (B) Western blot analysis of Iba-1 and GFAP in WT and TRAIL-R-/- mice following stereotaxic infusion of oligomeric Aβ1-42 or vehicle. (C) and (D) are respective densitometric analysis of the western blots. Data

are expressed as means ± SEM. One-way ANOVA and the Bonferroni post-hoc test were used to determine statistical significance. *p <0.05. N=5 animals for each group.

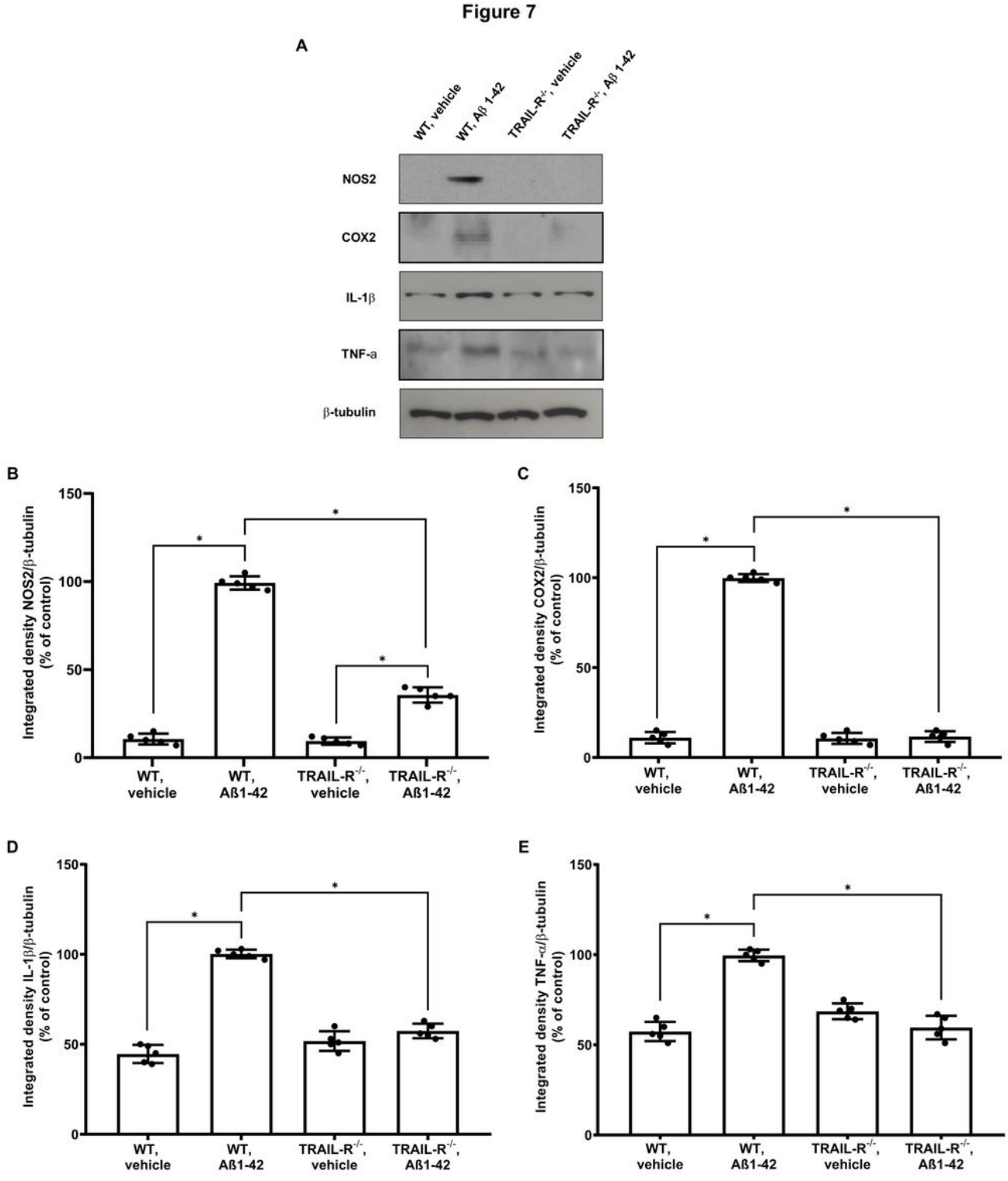

Figure 7

Inflammatory molecules expression is attenuated in TRAIL-R-/- mice treated with Aβ1-42. (A) Western blot analysis of NOS2, COX2, IL-1β and TNF-α in WT and TRAIL-R-/- mice following stereotaxic infusion of oligomeric Aβ1-42 or vehicle. (B) Densitometric analysis of western blots. Data are expressed as means ±

SEM. One-way ANOVA and the Bonferroni post-hoc test were used to determine statistical significance. *p <0.05. N=5 animals for each group.

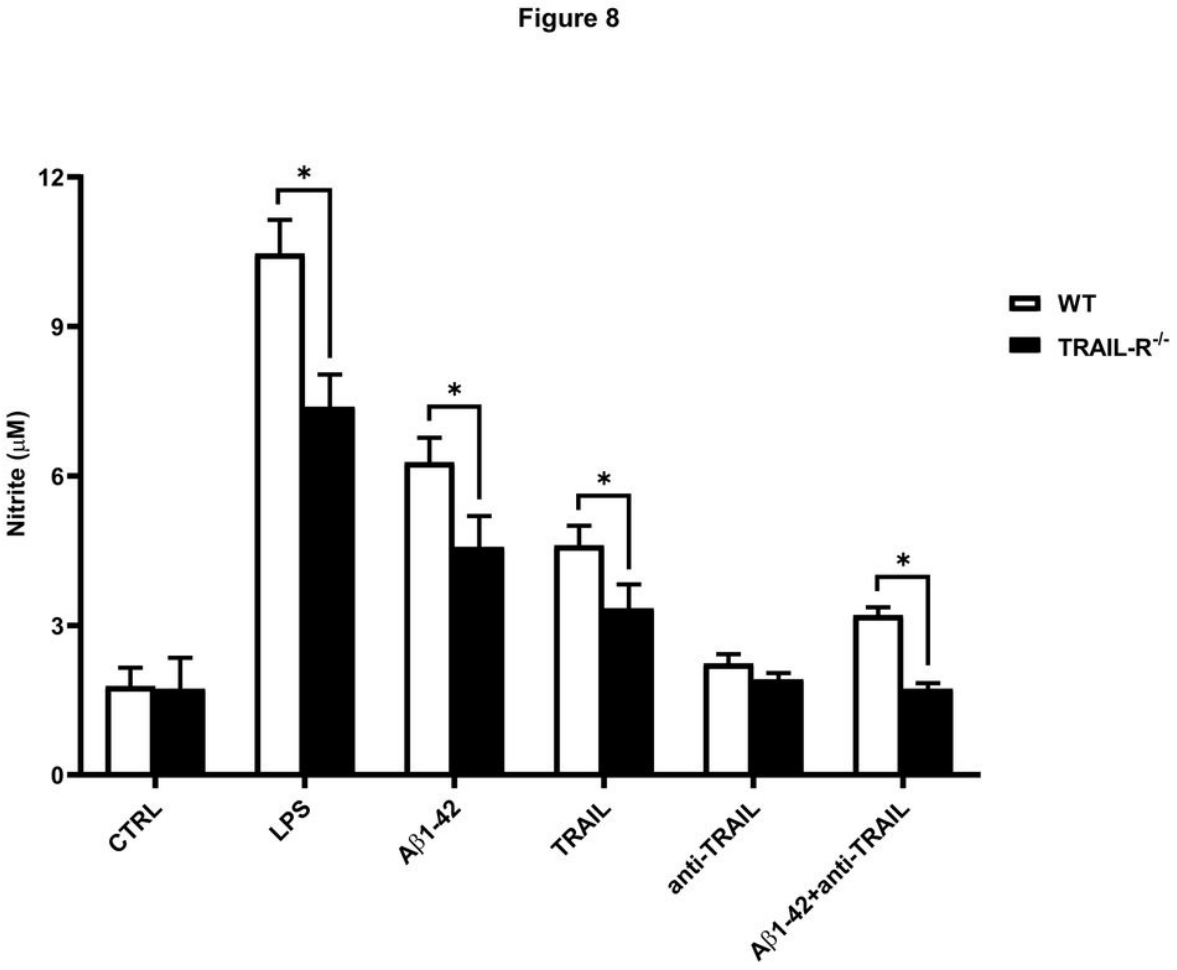

Figure 8

The lack of TRAIL-R2 is associated with a significative reduction of nitrite levels in the media of TRAIL-R-/- mouse primary hippocampal cells treated with Aβ 1-42. Nitrite levels in the media of embryonic hippocampal cells following 48h treatment with LPS (10μg/ml; positive control), Aβ1-42 (1 μM), TRAIL (100 ng/ml), anti-TRAIL (1 μg/ml) or Aβ1-42 plus anti-TRAIL. Data are expressed as mean ± SEM. One-way ANOVA and the Bonferroni post-hoc test were used to determine statistical significance. *p <0.05.