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Age-dependent changes on TGFβ1 Smad3 pathway modify the pattern of microglial cell activation

Juan E. Tichauer, Betsi Flores, Bernardita Soler, Laura Eugenín-von Bernhardt, Gigliola Ramírez, and Rommy von Bernhardt

Department of Neurology, Faculty of Medicine, Pontificia Universidad Católica de Chile. Marcoleta 391, Santiago, Chile.

Summary

Aging is the main risk factor for Alzheimer's disease. Among other characteristics, it shows changes in inflammatory signaling that could affect the regulation of glial cell activation. We have shown that astrocytes prevent microglial cell cytotoxicity by mechanisms mediated by TGFβ1. However, whereas TGFβ1 is increased, glial cell activation persists in aging. To understand this apparent contradiction, we studied TGFβ1-Smad3 signaling during aging and their effect on microglial cell function. TGFβ1 induction and activation of Smad3 signaling in the hippocampus by inflammatory stimulation was greatly reduced in adult mice. We evaluated the effect of TGFβ1-Smad3 pathway on the regulation of nitric oxide (NO) and reactive oxygen species (ROS) secretion, and phagocytosis of microglia from mice at different ages with and without *in vivo* treatment with lipopolysaccharide (LPS) to induce an inflammatory status. NO secretion was only induced on microglia from young mice exposed to LPS, and was potentiated by inflammatory preconditioning, whereas in adult mice the induction of ROS was predominant. TGFβ1 modulated induction of NO and ROS production in young and adult microglia, respectively. Modulation was partially dependent on Smad3 pathway and was impaired by inflammatory preconditioning. Phagocytosis was induced by inflammation and TGFβ1 only in microglia cultures from young mice. Induction by TGFβ1 was also prevented by Smad3 inhibition. Our findings suggest that activation of the TGFβ1-Smad3 pathway is impaired in aging. Age-related impairment of TGFβ1-Smad3 can reduce protective activation while facilitating cytotoxic activation of microglia, potentiating microglia-mediated neurodegeneration.

Keywords

Adult microglia; Aging; Alzheimer disease; beta amyloid uptake; cytokines; neuroinflammation; neurodegeneration; phagocytosis; signaling pathways

1. Introduction

Alzheimer's disease (AD) is characterized by the deposition of β-amyloid (Aβ) plaques and neurofibrillary tangles in brain parenchyma (Hardy & Selkoe, 2002), both of which are intimately associated with activated microglia and astrocytes. Glial cells have an important

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Corresponding author: Rommy von Bernhardt, MD, PhD Pontificia Universidad Católica de Chile, Faculty of Medicine. Department of Neurology, Marcoleta 391, Santiago, CHILE. Phone: 56-2-354 6936; Fax: 56-2-632 1924; rvonb@med.puc.cl.

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role in innate immunity, being the main producers of inflammatory mediators. Depending on the activation status, they secrete anti-inflammatory cytokines such as interleukin 10 (IL10) and transforming growth factor β (TGF β 1), pro-inflammatory cytokines such as interleukin 1 β (IL1 β), tumor necrosis factor α (TNF α) and interferon gamma (IFN γ), as well as reactive species such as nitric oxide (NO) and reactive oxygen species (ROS) including superoxide radicals (O $_2^{\cdot-}$) (von Bernhardt et al. 2010; von Bernhardt & Eugénin, 2012). In addition, there is solid evidence that glial cells participate in A β clearance (Alarcón et al. 2005; Paresce et al. 1996). When microglial cells are stimulated, production of inflammatory cytokines increases, whereas anti-inflammatory cytokines decrease (Ramírez et al. 2008).

TGF β 1 is a potent regulator of cytotoxicity and neuroinflammation in the nervous system. We have reported regulation of microglial cell cytotoxic activation by soluble factors, including TGF β 1 (Herrera-Molina & von Bernhardt 2005; Saud et al. 2005), secreted by astrocytes (Ramírez et al. 2005; Tichauer et al. 2007). Stimulation of hippocampal cultures with inflammatory mediators like lipopolysaccharide (LPS) and IFN γ induces increased levels and activation of TGF β 1 (Uribe et al. 2009), which in turn reduces microglial secretion of O $_2^{\cdot-}$ and NO (Saud et al. 2005; Herrera-Molina & von Bernhardt, 2005). Moreover, it has been demonstrated that TGF β 1 is increased in the cerebrospinal fluid (Rota et al. 2006) and plasma of AD patients (Motta et al. 2007).

There is evidence that TGF β 1 can be both beneficial and deleterious for AD. It has been implicated on the increased deposition of A β in blood vessels and meninges (Wyss-Coray et al. 1997), and the increased production of A β by astrocytes (Lesne et al. 2003) in APP/TGF β 1 transgenic mice. In contrast, other studies have shown that TGF β 1 has anti-amyloidogenic roles, reducing A β burden in the brain and inhibiting the formation of neuritic plaques, effects that appear to be mediated by activated microglia (Wyss-Coray et al. 2001). TGF β 1 secreted at the injury site can promote microglial cell recruitment, thus leading to the efficient removal of the noxious stimulus. Moreover, *in vitro* chemotaxis assays have shown that TGF β 1 induces microglial cell migration and modulates the chemotactic effect of nerve growth factor (NGF) (De Simone et al. 2007).

During aging, microglia show morphological changes and an exacerbated inflammatory response, changes that have been proposed to contribute to the onset of chronic neurodegenerative diseases (von Bernhardt et al. 2010). Moreover, aged microglia decrease their ability to phagocytose A β in comparison with young microglia (Floden & Combs, 2011). Anatomopathological studies of hippocampi from AD patients show that the expression of Smad3, one of the main effectors of TGF β 1, is diminished along with the existence of alterations in the subcellular localization of phosphorylated Smad2/3 proteins (Colangelo et al. 2002; Lee et al. 2006). The uncoupling of TGF β 1 signal transduction pathway could result in altered patterns of microglial activation and reduced clearance of amyloid, as is observed in aging and in AD.

Here we evaluate the effect of aging upon the regulation of microglial cell activation by TGF β 1-Smad3 pathway *ex vivo* after systemic inflammatory stimulation. We found that regulatory mechanisms depending on TGF β 1 signaling appear to be impaired in aging, favoring amyloid accumulation and microglial cell cytotoxic activation. As we will discuss (see Fig. 6), in young mice inflammation induces TGF β 1 signaling capable of regulating inflammatory activation and inducing A β uptake. In contrast, in adult mice, basal level of TGF β 1 signaling is elevated but it is not induced further by inflammatory activation. Persistent high levels of TGF β 1 appears to impair its beneficial effect.

2. Methods

2.1 Reagents

TGF β 1 was purchased from R&D, Inc. (Minneapolis, Minnesota, USA); LPS was from Sigma (St. Louis, Missouri, USA); Smad3 inhibitor SIS3 was from Calbiochem (San Diego, California, USA), primary antibodies, rabbit anti-Smad3, rabbit anti pSmad3 from Cell Signaling Technology (Danvers, Massachusetts, USA), lectin Alexa Fluor 568 (*Griffonia simplicifolia*), and GAPDH from Chemicon (Temecula, California, USA), mouse anti GFP from Santa Cruz Biotechnology (Dallas, Texas, USA), human anti-EEA1 was kindly donated by Dr. Alfonso Gonzalez (Faculty of Medicine, Pontificia Universidad Católica de Chile), fluorescent mounting medium from Dako Cytomation (Carpinteria, California, USA). Cell culture media, antibiotics and serum were purchased from HyClone (Thermo Scientific, HyClone Laboratories, Inc. Logan, Utah, USA).

2.2 Animal Models

WT mice (C57BL6/j) and transgenic mice MaFIA expressing Enhanced Green Fluorescent Protein (EGFP) under macrophage promoter MCSF (macrophage colony stimulating factor), were purchased from Jaxmice (Jackson Laboratory, Bar Harbor, ME, USA). All procedures followed the animal handling and bioethical requirements defined by the Pontificia Universidad Católica de Chile Ethics Committee in accordance with NIH guidelines. Animals were maintained at the institutional animal facility. They were anaesthetized before sacrifice. Two months (young) and twelve months old (adult) C57BL6/j mice received intraperitoneal (i.p.) injections with a single dose of PBS (vehicle) or LPS (0.5 mg/kg). Mice were sacrificed after 48 h by transcardiac perfusion with HANK'S under deep anesthesia with sodium pentobarbital (50 mg/kg body weight). After perfusion, brains were removed. Cortex was used to obtain microglial cell cultures and hippocampi were used for western blot analysis.

2.3 Adult microglia isolation and culture

Cultures were prepared according to von Bernhardt et al (2011). Briefly, brains were washed in cold HANK's solution and sequentially disaggregated passing the tissue sequentially through 150 and 60 μ m steel meshes. Disaggregated cortices were placed in a 50 mL conical tube, centrifuged at 170 g at room temperature (RT) for 10 min and resuspended in 10 mL of 10 % collagenase D in HANK's buffer plus 3 % fetal bovine Serum (FBS). Tubes were placed in an orbital shaker at 150 rpm and RT for 30 min. After incubation, tubes were filled with HANK's plus 3% FBS and centrifuged at 170 g at RT for 10 min. Supernatant was discarded and the cell pellet was separated through a discontinuous percoll gradient. Briefly, cells resuspended in 2 mL of 37% isotonic percoll (SIP) in HANK's were placed in a 15 mL conical tube containing 2 mL of 70 % SIP and 2 mL of 30 % SIP. Finally, 1 mL of HANK's solution containing 3% FBS was added above. The gradient was centrifuged at 1410 g at RT for 20 min. Microglial cells were collected from the 37 %/ 70 % interphase in a 50 mL conical tube filled with HANK's solution, gently mixed and centrifuged at 400 g at RT for 10 min. The recovered cells were grown in 24 well plaques in DMEM culture media with 10 % FBS and 20 % LADMAC cells conditioned media containing CSF1. Media was changed twice per week until cell confluence was reached.

2.4 Western Blot

Hippocampal tissue was homogenized in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and protease inhibitors). Samples were separated by electrophoresis in 12% poly-acrylamide gels and transferred to a nitrocellulose membrane. After transference, the membrane was treated with blocking buffer (0.05%

Tween 20, 5% milk in PBS) and then incubated with the primary antibody in blocking buffer: rabbit anti pSmad3, rabbit anti Smad3 (1:1000; Cell Signaling), and mouse anti GADPH (1:1000; Chemicon). The primary antibody was rinsed and the membrane was incubated with the corresponding horseradish peroxidase-conjugated secondary antibody: goat anti-rabbit or goat anti-mouse, in blocking buffer. Signals were detected by enhanced chemiluminescence substrate kit (PerkinElmer, Inc., Waltham, Massachusetts, USA) in accordance with the manufacturer's instructions. The molecular mass was estimated with BenchMark™ pre-stained protein Ladder (Invitrogen, Carlsbad, California, USA). Densitometry was done with the ImageJ NIH program.

2.5 Determination of Nitrites (NO₂⁻)

Nitrite (NO₂⁻), a stable downstream product of NO released to the cell culture medium, was determined by the Griess assay. Microglia (3×10^4 cells per 96 well) were exposed to the following conditions: control, 1 µg/mL LPS, or 1 µg/mL LPS + 2 ng/mL TGFβ1 for 48 h. To evaluate the relevance of the TGFβ1-Smad3 pathway, microglial cells cultures were pretreated for 1 h with 10 µM SIS3 (Smad3 inhibitor) prior to stimulation. For determination of nitrites, 50 µL of medium was mixed with 10 µL EDTA:H₂O 1:1 (0.5 M, pH 8.0) and 60 µL of Griess reagent (20 mg *N*-[1-naphthyl]-ethylenediamine and 0.2 g sulphanilamide dissolved in 20 mL of 5% phosphoric acid, w/v). Calibration curves were performed with 1–80 µM NaNO₂. Absorbency was measured at 570 nm in a microplate auto reader (ANTHOS 2010, Anthos Labtec Instruments (Salzburg, Austria).

2.6 Respiratory Burst Assay

The production of O₂ was assessed by the reduction of nitro blue tetrazolium (NBT) assay. Cells were cultured in fresh medium. Inflammatory activation of glial cells was elicited by addition of 1 µg/mL LPS at 37°C for 24 h. After treatment, culture medium was replaced with 1 mg/mL NBT, in phenol red-free DMEM/F-12 containing 1 mg/mL BSA. Respiratory burst was triggered with 150 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma) for 1.5 h. Next, glia were fixed with 100% methanol at room temperature. Cells were photographed with bright field microscopy in an inverted microscope Leica DMIL (Leica Microsystems, Wetzlar, Germany). Crystals were dissolved with 50 µL 1:1.15 2 M KOH/DMSO (Sigma) and absorbency was read at 645 nm in a microplate auto reader (ANTHOS 2010, Anthos Labtec Instrument).

2.7 Aβ Uptake Assay

To determine the ability of microglia to phagocytose non-fibrillar Aβ, we plated 5×10^5 microglia in complete DMEM/F12 on glass coverslips in 24-well plates. Microglia were incubated with 2 ng/mL TGFβ1 with and without pre-treatment with 10 µM SIS3 (a Smad3 signaling pathway inhibitor) for 1 h. After 48 h, cells were washed with DMEM/F12 and incubated with 1 µg/mL Aβ₁₋₄₀ Hilyte™ Fluor 488 in DMEM/F12 for 3 h. Cells were washed 2 times with 1mM Ca²⁺ in PBS and fixed with 4% paraformaldehyde at room temperature for 15 min. Fixed cells were permeabilized with 0.03% Triton X-100 in PBS for 15 min and the nucleus was stained with Hoechst for 10 min. Finally, glass covers were washed and mounted with Dako Cytomation fluorescent mounting medium. Analysis of the Aβ uptake was done on microphotographs of 10 fields per preparation acquired at 40X magnification by random sampling with an Olympus epifluorescence microscope, quantifying Aβ uptake (pixels per field/nuclei) and expressed as fold change compared with the young animal under control condition.

2.8 Immunofluorescence Labeling

Microglia were seeded on glass coverslip in 24-well plates at a density of 5×10^5 cells/well in complete DMEM/F12. Cells were fixed with 4% p-formaldehyde for 15 min, and permeabilized with 0.03% Triton X-100. Cells were incubated with mouse anti-GFP (1:100, Santa Cruz Biotechnology) antibody or human anti-EEA1 (1:200) at 4°C overnight. Secondary antibody conjugated to rhodamine was added for 2 h and cell nuclei were stained with Hoechst (Molecular probes, Inc., Eugene, Oregon, USA). Finally, cells were washed and mounted with DakoCytomation fluorescence mounting medium. Preparations were visualized in an inverted epifluorescence microscope (Leica).

2.9 TNF α and TGF β 1 Assay

TNF α and total TGF β were determined in 100 μ L of sera and mice hippocampus homogenized in cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and protease inhibitors). Samples were centrifuged at 14,000 g for 40 min. Supernatants were collected and protein content assayed by the BCA method. The levels of TNF α and TGF β were measured with anti-mouse TNF α and TGF β enzyme-linked immunosorbent assay (ELISA Ready-SET-Go!, eBioscience; San Diego, CA, USA).

2.10 Statistical Analysis

The *in vitro* data was expressed as mean $\pm$ SEM of at least 4-6 independent experiments in duplicate. Analyses were conducted with the GraphPad Prism (version 4.0) software (GraphPad Software INC., San Diego, CA, USA). We compared treated cells with their corresponding control conditions and analyzed them using a one-way analysis of variance (ANOVA) with Tukey-Kramer post-hoc test, Student-t test for western blots, and a two-way ANOVA test to compare different ages and treatment groups. For statistical analysis, a value of $p < 0.05$ was considered significant.

3 Results

3.1 Aging is associated with increased hippocampal cytokine levels and a decreased induction of TGF β 1 after *in vivo* stimulation with LPS

An age-dependent increase on cytokine levels was observed. Selected ages were 2 month (sexually mature young adult animals) and 12 month (adult animals at an age in which early stages of A β plaque formation and neurobehavioral impairment is observed). Basal hippocampal level for TNF α was increased by 45% ($P < 0.01$; $F = 1.79$; $df = 10$, Fig. 1A) and TGF β 1 was increased over 2-fold ($P < 0.01$; Fig. 1A $F = 8.47$; $df = 10$, Fig. 1A) in 12 months old animals compared with the levels observed at 2 months of age.

A systemic inflammatory status was generated by i.p. injection of LPS; stimuli known to induce synthesis of inflammatory cytokines both at the periphery and at the brain (Qin et al. 2007). A single i.p. injection of 0.5 mg / Kg LPS, induced a robust increase of blood TNF α (from 11.6 ± 4.3 pg / mL to $2,394 \pm 172$ pg / mL ($P < 0.001$; $F = 155.9$; $df = 4$, Fig. 1B) and TGF β 1 (a 20-fold increase, reaching levels in the order of 190 ± 52 pg/mL ($P < 0.001$; $F = 95.6$; $df = 4$, Fig. 1C) in young mice at 1 h post-injection. Increase of TGF β 1 in the brain was assessed at 48 h post-injection, showing a 35% increase after LPS injection ($P < 0.05$; $F = 2.89$; $df = 4$, Fig. 1D). Although TNF α basal levels were higher in adult mice than in the young mice, similar levels were reached at both ages after stimulation with LPS (from 27.5 ± 0.5 pg / mL to $2,419 \pm 669$ pg / mL ($P < 0.001$; $F = 605500$; $df = 5$, Fig. 1B). In contrast, in 12 months old mice, whereas TGF β 1 in plasma was also increased under basal conditions, the increase induced by LPS was mild, reaching a concentration in the order of 70 ± 52 pg / mg protein ($P < 0.05$; $F = 2.52$; $df = 4$, Fig. 1C). Moreover, hippocampal TGF β 1 was not

significantly increased by LPS (n.s; $F = 0.45$; $df=4$. Fig. 1D) above the basal level observed at 12 months. Our results show that the increase of TGF β 1 above its basal level in response to LPS was reduced in adult mice compared with young mice.

3.2 LPS induced Smad3 and pSmad3 in the hippocampus of young but not of old mice

Smad3 is the main effector of TGF β 1 (Schmierer & Hill, 2007). To explore whether this pathway is differently regulated in young and adult mice, we analyzed pSmad3 and Smad3 levels on the hippocampus of mice that received an i.p. injection of LPS 48 h before sacrifice. In order to be able to compare expression at different ages, Smad3 and pSmad3 were normalized according to the expression of the constitutive protein GAPDH. Smad3/GAPDH and pSmad3/GAPDH increased by $75 \pm 10\%$ ($P = 0.0031$; $F = 45.1$; $df=14$) and $60 \pm 10\%$ ($P < 0.005$; $F = 2.91$; $df=4$), respectively in young mice exposed to an inflammatory stimulus compared with control mice injected with vehicle (Fig. 2). In adult mice, basal levels of Smad3/GAPDH were 60% higher than in young mice, ($P < 0.01$; $F = 20.47$; $df=10$) whereas basal levels of pSmad3/GAPDH were around 50% ($P < 0.001$; $F = 1.4$; $df=6$). Inflammatory stimulation did not affect levels observed under un-stimulated conditions. These results indicate that even though basal levels of TGF β 1 increased with aging, the activation of Smad signaling in response to inflammatory stimulation appears to be abolished (Fig. 2; T-student unpaired test two tailed).

3.3 Aging and inflammation differentially affected NO and ROS secretion by microglia

For the assessment of adult microglia isolation (von Bernhardt et al. 2011), wild type C57BL/6j and transgenic MaFIA mice (Macrophage Fas Induced Apoptosis mice model) expressing green fluorescent protein (EGFP) under control of macrophage promotor *c-fms* were used. Isolated microglial cell colonies were observed after 1 week, reaching confluence after the third week of culture. Immunofluorescence against GFP and Iba-1 (Wako) or labelling with lectin (Isolectin B4, Sigma) allowed identification of microglia (Fig. 3A, GFP immunolabeling). Less than 3% of cells were positive for GFAP (data not shown). Microglia obtained through our protocol were of polymorphic shape, presenting both round shape, characteristic of activated cells, and ramified cells with long processes as described for surveillance state cells (Fig. 3A).

To evaluate microglial cell activation we characterized NO secretion by assessing its stable product nitrite (Fig. 3B) in the culture media by the Griess assay, and ROS production by the reduction of nitro blue tetrazolium (NBT assay; Fig. 3C). In microglial cell cultures obtained from young mice injected with PBS (vehicle injected mice) or inflammatory conditions (LPS injected mice), production of NO increased by 3.4 ± 1.1 fold ($P < 0.01$; $F = 7.67$; $df=23$) and 4.5 ± 1.5 fold ($P < 0.05$; $F = 20.4$; $df=16$) in response to LPS, compared with control cultures (Fig. 3B), respectively. A 2.5 ± 0.6 -fold ($P < 0.05$; $F = 6.4$; $df=21$) increase of ROS production was induced by LPS in microglia cultures obtained from young mice exposed to inflammatory conditions. In contrast, LPS did not induce ROS production in cultures obtained from vehicle injected mice (Fig. 3C).

In contrast to the activation pattern of microglia from young mice, LPS did not induce NO production in cultures obtained from adult mice, whereas ROS production was increased by 5.2 ± 1.5 fold ($P < 0.01$; $F = 8.46$; $df=17$) and 4.3 ± 1.1 fold ($P < 0.05$; $F = 6.45$; $df=15$) in cultures from mice injected with vehicle and inflammatory condition respectively (Fig. 3C).

3.4 Aging induced changes on phagocytosis profile on microglial cultures

Because there is evidence that TGF β 1 induce the removal of A β by the microglial cell line BV2 (Wyss-Coray et al. 2001), and there are increased basal levels of TGF β 1 in the hippocampus as animals age (Fig. 1A) the association of increased accumulation of A β

aggregates and aging appears to be a paradox. Therefore, we evaluated if the induction of microglial cell phagocytic activity by TGF β 1 could be affected by aging.

TGF β 1 increased A β phagocytosis by 2.71 ± 0.6 fold ($P < 0.05$; $F = 3.95$; $df = 16$) in microglial cell cultures obtained from young mice injected with vehicle. However, TGF β 1-induced phagocytosis showed a tendency to a partial reduction (1.5 ± 0.4 fold compared with control condition) when microglia were obtained from young mice previously exposed to LPS (Fig. 4A).

Basal phagocytosis of microglia obtained from adult mice was a 45% higher (without reaching statistical significance) than that of microglia from young mice. However, the induction of phagocytosis by TGF β 1 observed in microglia from young mice, was absent in adult mice microglia (Fig. 4A). Thus, our results show that both aging and systemic inflammatory preconditioning reduced the induction of phagocytosis by TGF β 1.

3.5 TGF β 1-induced A β uptake depends on the activation of the Smad3 pathway and inflammatory preconditioning

To assess the participation of TGF β 1-Smad3 pathway in the uptake of A β , microglia cultures were incubated with TGF β 1 for 48 h with or without pre-treatment with the Smad3 inhibitor SIS3. In microglia cultures from young mice injected with vehicle, inhibition of the Smad3 pathway by SIS3 decreased TGF β 1-induced A β uptake by 84.2% ($P < 0.05$; $F = 3.7$; $df = 16$), whereas in cultures derived from young mice exposed to inflammatory preconditioning, as well as in microglia cultures obtained from adult mice, TGF β 1 did not induce a significant increase in A β phagocytosis (Fig. 4B).

3.6 Phagocytosed A β co-localizes with early endosomes in microglial cells in culture

To evaluate whether TGF β 1 induced uptake of A β and changed its destination to specific intracellular compartments, co-localization of early endosome protein (EEA1) and A β was evaluated in microglia cultures (Fig. 4C). Control cultures obtained from mice injected with vehicle showed co-localization of EEA1 and A β predominantly in the periphery of cells. In cells treated with TGF β 1, co-localization of EEA1 with A β was less conspicuous and A β was more frequently observed in the perinuclear region; a localization that is consistent with destination to late endosomes for subsequent degradation.

In control cultures obtained from mice that received inflammatory preconditioning, co-localization of early endosome protein (EEA1) and A β at the cell periphery was more abundant than in cells obtained from vehicle injected mice, suggesting that microglia could be activated. TGF β 1-treated cells were larger in size than control microglia and showed A β in the perinuclear region that did not co-localize with EEA1. In general, A β co-localizing with EEA1 were reduced in microglia from animals preconditioned with LPS. Microglia exposed to TGF β 1 but pretreated with SIS3 were small, clustered and round shaped, and failed to show appreciable differences in EEA1 labeling or A β co-localization compared with TGF β 1-treated cells (Fig. 4C).

3.7 Production of ROS, but not NO by adult mice microglia was regulated by a TGF β -Smad3-dependent mechanism that was modified by inflammatory preconditioning

To assess the relevance of TGF β -Smad3 pathway for the modulation of NO and ROS production, microglial cell cultures obtained from young and adult mice injected with vehicle or LPS for 48 h were exposed to LPS or/and TGF β 1 in culture for 96 h, with or without 1h pre-treatment with the inhibitor of Smad3, SIS3.

TGF β 1 reduced LPS induced NO secretion by 79% ($P < 0.05$; $F = 6.32$; $df = 23$) in microglia cultures obtained from young animals injected with vehicle. However, TGF β 1 regulation was reduced for microglia obtained from young animals injected with LPS (Fig. 5). Inhibition of Smad3 by SIS3 had no effect on the regulation by TGF β 1 of NO secretion induced by LPS (Fig. 5). In contrast, LPS mediated induction of ROS production by microglia from young animals was only significantly increased by LPS when cells were obtained from animals previously pre-conditioned with LPS, increasing ROS production by 2.5 ± 0.6 fold ($P < 0.05$; $F = 6.4$; $df = 21$) compared with control cultures. LPS-induced ROS production remained elevated in presence of TGF β 1, reaching a 3.3 ± 0.7 fold increase (Fig. 5). ($P < 0.001$; $F = 12$; $df = 1$. Two-way ANOVA).

In microglia cultures from adult mice, stimulation of microglia with LPS had no effect on NO production (Fig. 5), whereas LPS treatment resulted in a robust increase of ROS production by 5.2 ± 1.5 fold ($P < 0.01$; $F = 9.7$; $df = 16$) and 4.3 ± 1.1 fold ($P < 0.05$; $F = 3.4$; $df = 15$) in microglia obtained from mice receiving preconditioning with vehicle and LPS respectively. TGF β 1 reduced by 68 % ($P < 0.05$; $F = 9.7$; $df = 16$) the induction of ROS production by LPS in cultures from vehicle injected mice. Inhibition of Smad3 by SIS3 partially decreased the modulator effect of TGF β 1 since ROS production was not statistically different from LPS treated cultures (Fig. 5). The reduction on ROS production induced by TGF β 1 was abolished in cultures obtained from mice preconditioned with LPS (Fig. 5). Inflammatory preconditioning resulted in a mild but significant increase on the production of ROS.

Thus, both aging and inflammatory preconditioning resulted in changes on NO and ROS production. In microglial cell cultures from young animals, inflammation induced an increase of NO secretion ($P < 0.05$; $F = 4.2$; $df = 3$; two-way ANOVA) and ROS production ($P < 0.01$; F_{012} ; $Df = 1$, two-way ANOVA). In contrast, microglia from adult mice showed no LPS preconditioning-dependent induction of NO or ROS production. However, inflammatory preconditioning abolished the reduction of ROS production by TGF β 1.

4. Discussion

Brain aging is associated with several changes, including an increase of inflammatory activity and oxidative stress, with elevated levels of inflammatory over anti-inflammatory cytokines in plasma and brain (von Bernhardt et al. 2010). It has been observed that microglia show a basally activated status during aging, which has been linked with neuronal damage, cognitive impairments and an increased susceptibility to neurodegenerative diseases, such as AD (Block et al. 2007; Hardy & Selkoe, 2002; Hauptmann et al. 2009; von Bernhardt, 2007; von Bernhardt et al. 2010)

Studies from our laboratory have shown that the activity of microglia *in vitro* is modulated by TGF β 1, which is produced mainly by astrocytes, decreasing NO and ROS production induced by LPS and IFN γ (Herrera-Molina & von Bernhardt, 2005; Herrera-Molina et al. 2012) and reducing neurotoxicity (Ramírez et al. 2005). However, it is known that TGF β 1 can have both beneficial and deleterious effects on various diseases (Lesne et al. 2003; Wyss-Coray et al. 1997; 2001).

Previous research have demonstrated the importance of Smad pathway, the main signal transduction pathway activated by TGF β receptors (Derynck & Zhang, 2003) on the regulatory and neuroprotective effects of TGF β 1; being involved in the induction of the quiescent phenotype of microglia within the CNS (Abutbul et al. 2012). Moreover, the inhibition of LPS-induced macrophage and microglial activation and the stimulation of A β phagocytosis by TGF β 1 is Smad3-dependent (Werner et al. 2000; Wyss-Coray et al. 2001;

Le et al. 2004; Tichauer & von Bernhardt, 2012). Recently, we have shown that TGF β 1, through the Smad3 pathway, induces glial cells to produce MKP-1, a phosphatase that exerts negative regulation on inflammatory activation, inhibiting A β -induced MAPK and NF κ B signaling and decreasing the production of TNF α and NO (Flores & von Bernhardt, 2012). Interestingly, this signaling pathway is impaired in the AD brain, inducing A β accumulation, A β -induced neurodegeneration and neurofibrillary tangle formation (Colangelo et al. 2002; Tesseur et al. 2006; Ueberham et al. 2006), even though TGF β 1 levels are elevated in cerebrospinal fluid of these patients (Rota et al. 2006; Zetterberg et al. 2004).

Here we show that both age and inflammatory status affect the amount and phosphorylation of Smad3 protein in mice hippocampus. In microglia cultures obtained from young (2 months old) mice, the inflammatory stimulus increased Smad3 levels in the hippocampus. In contrast, in adult (12 months old) animals, although Smad3 basal levels were increased compared with young animals, there was no further induction of Smad3 protein, and its phosphorylated fraction remained reduced, compared with young animal, after inflammatory stimulation. The increase in Smad3 levels observed in young mice could be explained by the increase in TGF β 1 levels induced by systemic inflammation (Wynne et al. 2010) that could activate mitogen activated protein kinase 1 (MAPK1) to induce Smad3 expression (Ross et al. 2007). However, in adult mice, increased basal levels of TGF β 1 due to age (Colangelo et al. 2002; Lukiw et al. 2004) could keep the high levels of Smad3, impairing the activation of TGF β 1-Smad3 signaling in response to inflammatory stimulation.

On the other hand, it should be noted that, besides Smad proteins, there are several additional signaling pathways activated by TGF β 1, including ERK, p38 and PI3K (Derynck & Zhang, 2003; Lee et al. 2007). Increased levels of TGF β 1 associated with a reduction of Smad signaling can result in an unbalance between the various pathways (Schmierer & Hill, 2007). Furthermore, considering that MAPKs and PI3K also participate in signal transduction of inflammatory activation, inhibition of the Smad pathway could result in the impairment of the modulatory effect of TGF β 1, favoring the cytotoxic activation of glial cells.

In contrast with our results indicating that TGF β 1 can decrease the accumulation of A β , there are several studies showing that increased levels of TGF β 1 are associated with increased amyloid angiopathy in the frontal cortex (Hamaguchi et al. 2005; Wyss-Coray et al. 1997). This effect appears to depend on the activation of astrocytes that stimulate the production of APP due to the presence of a TGF β 1 response element in the 5'UTR of APP (Docagne et al. 2004; Lesne et al. 2003). In addition, there are reports showing that transgenic mice hAPP/TGF β 1, in which astrocytes selectively express TGF β 1, develop a more accelerated accumulation of A β around vessels than the hAPP animal model by itself. However, these changes are also associated with a lower burden of A β in the parenchyma, which correlates with an increased microglia activation, suggesting that microglial cells could actively participate in the removal of A β (Wyss-Coray et al. 2001). Moreover, Tg2576 mice with a dominant negative TGF β 1-receptor II that blocks Smad2/3 signaling, show a conspicuous reduction of amyloid deposits in brain parenchyma (Town et al. 2008). Whereas this may seem initially contradictory with an anti-amyloidogenic role for TGF β 1, a possible explanation is that blockade of Smad2/3 could have occurred only in peripheral macrophages, sparing microglia. If that was the situation, microglia, the first line of brain defense would still be available for the active removal of A β (Sastre et al. 2006).

On the other hand, ROS production by microglia from adult animals was several folds higher than that observed in young animals, possibly because of the induction of the enzyme NADPH. In contrast, LPS did not induce NO in these animals, probably because of the lack of induction of iNOS or the increased formation of peroxynitrite in response to the increased

oxidative stress (Brown, 2007). Moreover, whereas in young mice LPS induces iNOS through stimulation of toll like receptors (TLR), which signal through widely studied pathways such as ERK, p38 and NF- κ B (Lee et al. 2007; Yu et al. 2002), the lack of induction in adult mice could be explained by the down regulation of TLR, or by changes in the signaling pathways activated by binding of LPS to TLR favoring one upon other. Further research would be necessary to explain these age-dependent changes.

Another main finding in this work was that systemic inflammatory stimulation and aging inhibited the modulation of NO and ROS production and the induction of A β phagocytosis by TGF β 1. Depending on age, microglia stimulated with LPS predominantly show induction of NO or ROS production in young and adult mice, respectively. Induction of both NO and ROS was prevented by TGF β 1 in microglial cell cultures obtained from young mice injected with vehicle, but modulation was abolished on microglial cell cultures obtained from animals that received inflammatory stimulation. Therefore, inflammatory stimulation elicited by systemic LPS induced a different response depending on age, becoming more oxidative, and for that reason, potentially more cytotoxic in aged animals. Moreover, TGF β 1 induced A β phagocytosis in a Smad3-dependent manner and only in young animals without LPS treatment. These results could explain, at least in part, the susceptibility to cognitive impairments and neurodegenerative diseases observed with aging.

A working model is shown in Figure 6. In young mice, LPS increases the expression of Smad3 and pSmad3 and induces the production of NO, which is inhibited by TGF β 1. Moreover, TGF β 1 induces A β uptake by a Smad3-dependent manner. On the other hand, in adult mice, basal levels of Smad3 and pSmad3 are elevated and they are not further increased by administration of LPS. TGF β 1 inhibits the production of ROS induced by LPS but cannot induce A β uptake. Increased TGF β 1-Smad3 activity on microglia associated with aging appears to impair the beneficial effects of TGF β 1, increasing ROS production and A β accumulation.

4.1 Our results suggest that TGF β 1-Smad3 signaling pathway is impaired in aging and in conditions of persistent inflammatory activation, becoming unable to modulate the activity of microglial cells. This effect, together with the predominant oxidative response observed in aged animals, could be responsible for a predominantly cytotoxic activation and relevant for the understanding of molecular mechanisms underlying neurodegenerative disorders such as AD. Therefore, additional studies should be done in order to determine if modulation of the function of microglia and TGF β 1-Smad3 signaling could represent a therapeutic strategy for AD or other age-related neurodegenerative processes. Furthermore, the age-dependent differences in the activation of microglia we described indicate that neonatal cells are clearly suboptimal experimental models for the study of biological mechanisms of neurodegenerative diseases. Extreme care should be used, especially for the search for therapeutic tools.

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Research Highlight

Age-dependent impairment of TGF β 1-Smad3 signaling reduces protective functions of microglia favoring cytotoxic activation and potentiating neurodegeneration

Figure 1.

Age dependent changes of inflammatory cytokine levels in the hippocampus and plasma of adult mice. TNFα and TGFβ1 were determined by ELISA. **A.** TNFα and TGFβ1 levels were increased in the hippocampus of 12 month old mice compared with 2 month old mice under non-stimulated conditions. **B-D.** Age dependent changes on *in vivo* plasma and hippocampal level of cytokines induced by i.p. injection of LPS. Young and adult mice were injected with a single dose of i.p. vehicle (PBS) or 0.5 mg / kg LPS (LPS). **B.** The concentration of TNFα in plasma was assessed at 1 h post-injection. TNFα showed a similar increase in young and adult mice in response to LPS injection. The concentration of TGFβ1 in **C.** plasma and **D.** hippocampus was assessed at 48 h post-injection. Plasma levels of TGFβ1 after LPS treatment were higher in young than in adult mice. In the hippocampus of young animals, LPS induced a moderate increase of TGFβ1. However, the increased basal levels of TGFβ1 observed in adult animals were not significantly increased by LPS treatment. Data correspond to the mean ± SEM of 5 independent experiments, * P < 0.05, ** P < 0.01, *** P < 0.001 compared with control condition, ANOVA with Tukey-Kramer post-hoc.

Figure 2.

Effect of i.p administration of LPS on the expression of Smad3 and pSmad3 in the hippocampus of young and adult mice. Immunoblot for Smad3 and pSmad3 were performed 48 h after young and adult mice received vehicle (PBS) or LPS. GAPDH immunoblot was used as load control in order to have normalized expression of Smad Protein at different animal ages. Smad3/GAPDH and pSmad3/GAPDH expression at the protein level increased in young but not in adult animals injected with LPS. Data correspond to the mean $\pm$ SEM of 4-6 independent experiments * P < 0.05, ** P < 0.01, *** P < 0.001 T-student Unpaired test.

Figure 3.

Effect of age and inflammation on NO and ROS production induced by LPS in adult microglial cells in culture. A. Cell identity and purity of microglial cell cultures derived from 2 and 12 months old mice were assessed by immunofluorescence against GFP in glial cultures obtained from Mafia transgenic mice expressing EGFP under the specific c-fms promoter. Arrowheads show elongated cells, characteristic of surveillance microglia, whereas arrows show round shaped cell bodies consistent with an activated phenotype. Bar = 50 μ m. Young and adult mice received a single i.p. injection of vehicle (PBS) or LPS (0.5 mg/Kg animal). Microglia cultures were established 48 h post injection, and production of B) NO and C) ROS induced by LPS were evaluated by the Griess assay and NBT assay, respectively, after cultures were exposed or not to 1 μ g / mL LPS for 48 h. Data correspond to the mean $\pm$ SEM of 5 independent experiments in duplicate, * P < 0.05, ** P < 0.01, ANOVA with Tukey's post hoc). Whereas basal levels of NO and ROS were similar at both ages, inflammatory activation of cultures predominantly induced NO production in young animals and ROS production in microglia obtained from aged mice.

Figure 4.

Effect of age and *in vivo* inflammatory pre-conditioning on TGFβ1-induced Aβ uptake by microglia in culture. Microglia cultures were obtained after mice received an i.p. injection of PBS (vehicle) or 0.5 mg / Kg LPS (LPS). **A,B.** Cell cultures were treated with 2 ng / mL TGFβ1 for 48 h and phagocytosis was assayed with HiLyte™ Fluor488-Aβ for 3 h. Nuclei were stained with Hoechst. Analysis of Aβ uptake was done by quantifying Aβ uptake (pixels per field/nuclei) and expressed as fold change compared with the young animal under control condition. **B, C.** Effect of Smad3 inhibition on Aβ uptake induced by TGFβ1 in microglial cell cultures from mice exposed to inflammatory stimuli. Cell cultures were treated with TGFβ1 with or without pre-treatment with 10 μM SIS3. Data correspond to the

mean $\pm$ SEM of 5 independent experiments in duplicate, For **A,B**: ANOVA with Tukey's post hoc. *P <0.05, treatment vs. control (F= 3.9; df=16) and # P <0.05 (F= 3.7; df=16), comparison with TGF β 1 treatment. No differences were observed at 12 month old animals (df=11; with F= 0.68 and 0.46, respectively) **C**. Effect of TGF β 1-Smad3 on the localization of A β in early endosomes in microglial cells from young mice exposed to inflammatory conditions. Microglia from young mice injected with vehicle or LPS were exposed to 2 ng/mL TGF β 1 for 48 h with or without pre-treatment with 10 mM SIS3 for 1 h, and then exposed to HiLyte™ Fluor-A β ₁₋₄₀ (red staining) for 3 h. Cells were fixed, permeabilized for immunofluorescence for the endosomal marker EEA1. Bar = 50 μ m.

Figure 5.

Effect of TGFβ1 and involvement of TGFβ1-Smad3 pathway in NO and ROS production by microglia in aging. Microglia cultures were established 48h after mice were injected with vehicle (PBS) or LPS. Microglia were exposed to 1 μg/mL LPS or 1 μg/mL LPS + 2 ng/mL TGFβ for 96 h, with or without pre-treatment with 10 μM SIS3 for 1 h. NO and ROS production were determined after 96 h. Data correspond to the mean ± SEM of 5 independent experiments done in triplicate. *, Treatment vs. control, * P < 0.05, ** P < 0.01, *** P < 0.001; # P < 0.05, compared with LPS treatment, and &, P < 0.05, && P < 0.01, comparing microglia obtained from mice injected or non-injected with LPS. ANOVA with Tukey's post hoc intra-group. &, Vehicle vs. LPS, two-way ANOVA with Bonferroni post test analysis revealed a significant effect of inflammatory preconditioning upon response to Nitrites and ROS production induced by LPS in 2 month old animals (P < 0.05; F = 4; df = 1) Other p; F and df values are indicated in the result section).

Figure 6.

TGFβ1-Smad3 pathway and activation of aging microglia. LPS increased the expression of Smad3 and pSmad3 in young mice. TGFβ1 was able to decrease the production of NO induced by LPS, an effect that was independent of Smad3. TGFβ1 induced Aβ uptake by a Smad3-dependent manner. In adult mice, basal levels of Smad3 and pSmad3 were elevated. However, they were not further induced by administration of LPS. TGFβ1 inhibited production of ROS induced by LPS at least partially in a Smad3-dependent manner. Basal levels of Aβ uptake were increased in microglia obtained from adult animals compared with those of young mice, but TGFβ1 did not induce further uptake of Aβ. Increased TGFβ1-Smad3 activity on microglia associated with aging appears to impair the beneficial effect of TGFβ1, favoring cytotoxicity depending on the increased production of ROS and the accumulation of Aβ secondary to its decreased removal.