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Differential regulation of macrophage phenotype by mature and pro-nerve growth factor

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ABSTRACT

To characterize the role of neurotrophin receptors on macrophages, we investigated the ability of nerve growth factor (NGF) and its precursor, proNGF, to regulate human macrophage phenotype. The p75 neurotrophin receptor ($p75^{NTR}$) and TrkA were concentrated within overlapping domains on membrane ruffles. NGF stimulation of macrophages increased membrane ruffling, calcium spiking, phagocytosis and growth factor secretion. In contrast, proNGF induced podosome formation, increased migration, suppressed calcium spikes and increased neurotoxin secretion. These results demonstrate opposing roles of NGF and proNGF in macrophage regulation providing new avenues for pharmacological intervention during neuroinflammation.

1. Introduction

Macrophages are dynamic cells that can express a wide range of phenotypes driven by external cues. The phenotypes range from strong inflammatory responses designed for elimination of invading pathogens to anti-inflammatory, protective and wound healing activities essential for tissue repair. To highlight the different functional states, many studies have focused on characterizing the phenotypes of macrophages as classically or alternatively activated based on their receptor composition, secretion profiles, morphology and response to external cues. Classically activated inflammatory macrophages have been shown to arise from interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and lipopolysaccharide (LPS) stimulation leading to secretion of pro-inflammatory cytokines and chemokines, often with accompanying tissue damage. Alternative activation of macrophages is stimulated by interleukin-4 (IL-4), IL-10, transforming growth factor-β (TGF-β), or IL-13 and leads to the secretion of anti-inflammatory cytokines, chemokines, growth factors and other reparative factors (Laskin et al., 2011). In addition to these well characterized stimuli, macrophages in various tissues can be regulated by a wide array of external cues causing phenotypes that may intertwine these subgroups through mechanisms that are not fully understood.

A potentially important but poorly explored set of cues may be neurotrophic factors. Although it has been over two decades since the first studies identified neurotrophin interactions within the immune system (Levi-Montalcini et al., 1996), our knowledge of their functions is limited. Multiple studies have documented expression of various neurotrophins and their receptors in macrophages suggesting that they may play a role in control of the innate immune system (Elkabes et al., 1998; Nakajima et al., 1998; Dowling et al., 1999; Barouch et al., 2001; Levanti et al., 2001; Aronica et al., 2004; Artico et al., 2008; Samah et al., 2008; Tonchev et al., 2008). Relatively, few studies have looked closely at the functions of these receptors on macrophages. Most information regarding the functions of the neurotrophin receptors comes from studies in the nervous system where neurotrophins are important factors for development, maintenance, survival and differentiation of neurons (Reichardt, 2006). The neurotrophin family includes nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). Neurotrophins bind to tyrosine protein kinases known as tropomyosin receptor kinase (Trk) receptors, TrkA (NGF), TrkB (BDNF and NT-4), and TrkC (NT-3) with high affinity. An additional member of the neurotrophin receptor family, the p75 neurotrophin receptor ($p75^{NTR}$) is a member of the tumor necrosis receptor family and binds all neurotrophins with low affinity. The neurotrophin receptors function as homomeric or heteromeric complexes, providing opportunities for various signaling actions. The $p75^{NTR}$ in particular can interact with any of the Trk

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receptors where it facilitates receptor activation by increasing the affinity of mature neurotrophin binding.

The neurotrophins are synthesized as precursors (pro-neurotrophins) that must be processed by proteolysis to form the mature protein. All pro-neurotrophins (proNGF, proBDNF, proNT3, proNT4) bind the $p75^{NTR}$ when it associates with alternative co-receptors such as sortilin. Signaling of mature and pro-neurotrophins through their respective receptors often have opposing effects in target cells. Mature neurotrophin signaling has been associated with neuronal survival, growth and differentiation while proNGF signaling often leads to neuronal degeneration and apoptosis (Ibanez, 2002; Hempstead, 2009). These differences have led to the hypothesis that the balance of pro-neurotrophins versus mature neurotrophins may regulate the course of neurodegenerative diseases (Hempstead, 2009). Regulation of macrophage and microglial functions by neurotrophins may be particularly important in the nervous system where neurotrophin expression is high. In addition to neurons macrophages also secrete neurotrophins. Neurotrophin mRNA expression has been documented in microglial/macrophages in multiple sclerosis plaques (Dowling et al., 1999) as well as HIV-infected macrophages (Samah et al., 2009). NGF has been shown to increase CXCR4 mediated migration of macrophage precursor cells, monocytes (Samah et al., 2008) and to induce the secretion of plasminogen and urokinase-type plasminogen activator from microglia (Nakajima et al., 1998). No studies have yet compared the functional activation of these receptors by pro- versus mature neurotrophins. The following studies were designed to further characterize the expression of neurotrophin receptors on human monocytes and monocyte-derived macrophages (hMDMs) and determine the functional role of mature versus pro-neurotrophins. We show that monocytes and macrophages express both $p75^{NTR}$ and TrkA within the same membrane domains and exhibit very different phenotypes in response to mature NGF and proNGF.

2. Materials and methods

2.1. Isolation and culture of human monocyte-derived macrophages

Human buffy coat leukocytes were purchased and shipped within 24 h after blood draw from healthy donors at the New York Blood Center (http://nybloodcenter.org/), a non-profit organization for the collection and distribution of blood for clinical and research purposes. All research use was screened by the center and no personal identifiers were sent with the shipment. Blood was diluted 1:1 with phosphate buffered saline (PBS) and was layered on top of Ficoll-Paque (GE Healthcare 17-1440-03). Blood/Ficoll-Paque was centrifuged at 500 ×*g* for 25 min and the peripheral blood mononuclear cells (PBMCs) were collected from the PBS/Ficoll-Paque interface. PBMCs were washed in red blood cell lysis buffer (Sigma R7757) to remove any red blood cell contamination. PBMCs were centrifuged at 450 ×*g*, the supernatant aspirated and the pellet re-suspended in Dulbecco's modified eagle medium (DMEM) with high glucose, 10% fetal bovine serum (Gibco 160000-044) and 20 μg/ml gentamicin (Gibco 15750-60). Cells were aliquoted into low adhesion 6 well plates (Corning 3471) at a density of approximately 10^7 cells/well. PBMCs were cultured for 5–7 days to allow monocyte attachment. Remaining white blood cells were washed, from the plate yielding a pure monocyte/macrophage culture. The adherent cells were differentiated into monocyte-derived macrophages (hMDMs) using human GM-CSF (15 ng/ml) in complete DMEM for one week. Monocyte experiments were carried out within 1 h of PBMC isolation to prevent cell attachment.

2.2. Primary cultures of rat forebrain

All animal work was done in accordance with NIH animal welfare guidelines and was approved by the University of North Carolina-Chapel Hill Institutional Animal Care and Use Committee (approval number 14-147.0). Timed gestational embryonic day 9 (E9) pregnant female Long–Evans rats were delivered from Charles Rivers and allowed to rest in UNC animal husbandry until the time of experiments. At gestational day E17, rats were sacrificed by anesthetizing with isoflurane until breathing and heart stopped. The uterus was removed, rinsed briefly in 70% ethanol and placed in HEPES-buffered Hank's balanced salt solution (HBSS) on ice. The brain was removed from each fetus, extensively washed, and the cortex/hippocampus was dissected from each brain and cleaned of dura-arachnoid membrane and visible vessels. The tissue was transferred to a 15 ml tube containing 5 ml calcium–magnesium free-HBSS + 2.4 U/ml dispase + 2 U/ml DNase I and incubated for 25–30 min at 36 °C. Tissue was triturated and allowed to settle for 2 min. The suspended cells were transferred to a 50 ml culture tube containing 25 ml of minimum essential medium (MEM) with glutamine + 10% fetal bovine serum + 20 μg/ml gentamicin. After several rounds of trituration in 2–3 ml fresh calcium–magnesium free HBSS, dissociated cells were seeded at a density 20,000 cells/cm^2 on poly-D-lysine-treated coverslips for imaging and staining or 50,000–100,000 cells/cm^2 in 100 mm plastic dishes for Western blots. After 24 h, cultures were transferred to Neurobasal medium with B27 supplement. The resulting cultures were >95% neurons at day 4 after seeding.

2.3. Immunostaining

Differentiated hMDMs grown on poly-D-lysine coated coverslips were transferred to DMEM containing 1% FBS and stimulated for 1 or 24 h using three different conditions: NGF human recombinant protein (100 ng/mL, Sigma N1408), proNGF human recombinant protein targeted to high affinity sites (1 ng/ml, Alamone N-280), or vehicle. The cells were gently washed and fixed in 2% paraformaldehyde in PBS. Cells were washed 3× in PBS and incubated in 3% normal goat serum for 1 h. Incubation of primary antibodies was carried out overnight at 4 °C. Cells were stained using antibodies to: $p75^{NTR}$ (Millipore cat #07-476, 1:500), TrkA (Santa Cruz cat #SC-80961, 1:500), TrkB (Millipore 07-225, 1:500), TrkC (Santa Cruz SC14025, 1:500) and sortilin (Millipore AB9712, 1:500). Cells were washed in PBS and incubated with species specific secondary antibodies conjugated to Alexa 488, 568 or 593 (Molecular Probes) in the dark for 1 h at room temperature. Coverslips were then mounted using Fluoromount (Southern Biotech 0100-01) and digitally imaged on an Olympus XI71 microscope.

Analysis of stain intensity and morphology for individual cells or regions of interest within the cells was accomplished using Metamorph software. Co-localization of $p75^{NTR}$ foci with TrkA or sortilin staining was analyzed by staining one receptor red and the other green followed by thresholding by intensity of stain, computer identification of stained objects and then documentation of each object's central X/Y coordinate for each wavelength. Objects with X/Y coordinates that overlapped within 0.46 μm were scored as co-localized.

2.4. F-actin and live-dead stains

F-actin was stained using Alexa488 phalloidin (1:50, Molecular Probes) to show structural changes. Podosomes were visible in the hMDM as small, intensely fluorescent puncta whereas ruffles appeared as moderate to brightly stained extensions of the membrane. Cell viability was assessed by incubating cells with the live cell stain, calcein AM (1 μM, Invitrogen/Molecular Probes) and the dead cell nuclear stain, ethidium homodimer (1 μM, Invitrogen/Molecular Probes), for 30 min at 36°. Cells were washed with aCSF and imaged live. Healthy macrophages with bright green fluorescence were counted. The unstained "ghosts" of dead cells were often visible and were counted separately. The number of ethidium stained nuclei was counted to quantify dead cells with multinucleated cells counted as a single cell. Some ethidium stained cells were observed floating on the surface which were difficult to quantify. Thus, the count of live cells was preferentially used as the best index of cell survival. Some cultures were fixed with 2%