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ProNGF mediates death of Natural Killer cells through activation of the p75NTR-Sortlin complex

Mary-Louise Rogers^{1,*}, Sheree Bailey^{2,3}, Dusan Matusica¹, Ian Nicholson⁴, Hakan Muyderman⁵, Promila C Pagadala⁶, Kenneth E Neet⁶, Heddy Zola⁴, Peter Macardle^{2,3}, and Robert A Rush¹

¹Department of Human Physiology, Allergy and Arthritis, School of Medicine, Flinders University, GPO Box 2100 Adelaide 5001

²Department of Immunology, Allergy and Arthritis, School of Medicine, Flinders University, GPO Box 2100 Adelaide 5001

³SA Pathology GPO Box 14 Rundle Mall, SA 5000

⁴Women's and Children's Health Research Institute, King William Rd, North Adelaide 5006

⁵Medical Biochemistry, School of Medicine, Flinders University, GPO Box 2100 Adelaide 5001

⁶Department of Biochemistry and Molecular Biology, Rosalind Franklin University of Medicine and Science, Chicago, USA

Abstract

The common neurotrophin receptor P75NTR, its co-receptor sortilin and ligand proNGF, have not previously been investigated in Natural Killer (NK) cell function. We found freshly isolated NK cells express sortilin but not significant amounts of P75NTR unless exposed to interleukin-12 (IL-12), or cultured in serum free conditions, suggesting this receptor is sequestered. A second messenger associated with p75NTR, neurotrophin-receptor-interacting-MAGE-homologue (NRAGE) was identified in NK cells. Cleavage-resistant proNGF123 killed NK cells in the presence of IL-12 after 20h and without IL-12 in serum free conditions at 48h. This was reduced by blocking sortilin with neurotensin. However, we found no evidence that NK cells produce endogenous proNGF or NGF. We conclude that proNGF induced apoptosis of NK cells may have important implications for limiting the innate immune response.

Keywords

ProNGF (pro Nerve Growth Factor); Natural Killer (NK) cells; Apoptosis; Sortilin; TNFRSF (tumour necrosis factor receptor super family)

* Address for correspondence, Dr Mary-Louise Rogers, Centre for Neuroscience, Department of Human Physiology, School of Medicine, Room 6E136, Flinders Medical Centre, Bedford Park, 5042, South Australia, Australia. Ph: 61 88204 5320 Fax 61 88204 5768; mary-louise.rogers@flinders.edu.au.

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1. Introduction

Tumor necrosis factor receptor super family (TNFRSF) ligands are crucial to Natural Killer (NK) cell development, ability to kill and their cross talk to other immune cells (Kashii et al., 1999; Vivier et al., 2008; Zingoni et al., 2004). There is growing evidence that TNF family receptors play a role in death of NK cells. Cytokine priming by, for example, interleukin-12 (IL-12) can render NK cells susceptible to activation induced cell death (AICD) and this involves the release of interferon-gamma (IFN- γ) which initiates autocrine release of tumor necrosis factor- α (TNF- α) that kills the cell (Ross and Caligiuri, 1997). In addition, upon tumor cell contact, NK cells release the TNF family ligand FasL which kills the tumor cell and also the NK cell itself through the engagement of Fas by FasL (Poggi et al., 2005). A non-autocrine method of NK cell killing involving TNFR and ligands also occurs whereby tumors produce soluble TNFRSF18, a glucocorticoid-induced TNF-related protein (GITR) ligand that binds GITR on NK to and kills the cells (Baltz et al., 2008; Gurney et al., 1999; Kwon et al., 1999). One TNFRSF receptor that has not been examined is the p75 neurotrophin receptor (p75NTR). In both the nervous and immune systems the activities of neurotrophins are exerted by binding two classes of receptors, p75NTR and the tyrosine kinase (trk) receptors which belong to the tropomyosin family of receptors (Schweigreiter, 2006). P75NTR binds all neurotrophins while trkA, trkB and trkC selectively bind nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and neurotrophin 3 (NT3) respectively. NGF has been demonstrated to have diverse functions in the nervous system including neuronal migration, synaptogenesis, cell death, axonal elongation, myelination, neuronal differentiation and neuronal survival (Bronfman et al., 2007; Schweigreiter, 2006).

In the immune system, NGF promotes survival and activation of eosinophils, induces differentiation in B-lymphocytes, augments cytokine synthesis in T cells, and increases proliferation, differentiation, and production of various mediators in mast cells (Ehrhard et al., 1993; Kobayashi et al., 2002; Matsuda et al., 1991; Otten et al., 1989). NGF affects functional properties such as chemotaxis and phagocytosis in neutrophils and granulocytes (Boyle et al., 1985; Gee et al., 1983; Kannan et al., 1991), and induces proliferation and differentiation of monocytes through the trkA receptor (Ehrhard et al., 1993; Kannan et al., 1993). However, NGF has not been examined in NK cells.

NGF induces signalling pathways resulting in cell growth by binding as a dimer to two trkA receptors, but these signals are enhanced if NGF as a dimer instead binds one trkA and one p75NTR receptor (see Schweigreiter, 2006 for review). However, p75NTR contains a death domain and cell death signals arise from NGF binding to p75NTR (Chao et al., 1998; Nykjaer et al., 2005). Similar to other members of the TNFRSF, numerous second messengers are associated with p75NTR death signaling, including the neurotrophin-receptor-interacting-MAGE-homologue (NRAGE; see Salehi et al., 2000; Schweigreiter, 2006 for review).

NGF is synthesized as pre-pro NGF and after the signalling peptide is split from the molecule in the endoplasmic reticulum, the proNGF is either cleaved intracellularly to mature NGF, or transported to the plasma membrane and released, where it may be processed to the mature form (Lee et al., 2001). Furin and proconvertases have been implicated in intracellular cleavage, whereas metalloproteinases such as MMP9 mediate extracellular cleavage (Bruno and Cuello, 2006; Kennedy et al., 2007). proNGF has been shown to be involved in cell death (Jansen et al., 2007; Nykjaer et al., 2004). The receptor sortilin was demonstrated to partner p75NTR as a membrane bound co-receptor for proNGF induced neuronal cell death. It also appears to be kinetically favourable for the mature and pro domains of proNGF to bind p75NTR and sortilin respectively. In addition, proNGF has

increased affinity for p75NTR-sortilin over sortilin alone. ProNGF mediates neuronal cell death through binding this complex and blocking sortilin prevents this action (Nykjaer et al., 2004).

Sortilin belongs to the mammalian Vps10p family of receptors, that includes SorLA, and SorCS1-3, once considered as having only intracellular sorting functions (Willnow et al., 2008). It was initially described as neurotensin receptor 3 (NTR3) (Petersen et al., 1997). Sortilin was first identified as a 95kDa intracellular sorting receptor that directs movements of newly synthesized proteins such as the unprocessed form of nerve growth factor (proNGF), neurotensin, lipoprotein lipase and pro-brain-derived neurotrophic factor (pro-BDNF; (Lin et al., 1997; Mazella et al., 1998; Munck Petersen et al., 1999; Nyborg et al., 2006; Nykjaer et al., 2004; Teng et al., 2005). Sortilin was also found to function as a larger 100kDa receptor for these proteins once exposed on the cell surface, usually in concert with another receptor (Jansen et al., 2007). There is very little known about the distribution of sortilin in the immune system and proNGF is not known to function in immune cells. Sortilin mRNA has been identified in B cells and shown to be involved in trafficking BDNF and proBDNF in these cells (Fauchais et al., 2008). Blocking sortilin had an anti-apoptotic effect in some B cell lines exposed to BDNF, but not in isolated primary B cells. In this current study we report that the TNFR p75NTR and its co-receptor sortilin are present on NK cells and that proNGF induces apoptosis in un-stimulated primary NK cells in the presence of interleukin-12.

2. Materials and Methods

2.1 Flow cytometry

Blood was drawn from healthy adult volunteers and peripheral blood mononuclear cells were separated using Lymphoprep (Axis-Shield Proc As). The antibody panel used to determine mononuclear cell populations comprised CD3 (T cells), CD4 (helper T cells), CD8 (CD8 positive T cells), CD19 (B cells), CD45 (leukocytes), CD14 (monocytes), CD16 (NK cells) and CD 56 (NK cells), (BD Biosciences). Rabbit anti-sortilin to the whole extracellular domain was kindly provided by Professor Claus Munck Petersen (Munck Petersen et al., 1999). The monoclonal anti-p75NTR from MLR2 hybridoma was purified as previously described (Rogers et al., 2006). Monoclonal p75NTR antibodies from ME20.4 and 8211 clones were from Biosensis and Millipore respectively and NGFR5 from BD Biosciences. Anti-human trkA monoclonal antibody was purchased from R&D systems (clone 165126) or kindly provided by Prof Uri Saragovi (McGill University, Canada; (LeSauter et al., 1996)). Secondary anti-rabbit FITC or Alexa-488 conjugate were from Invitrogen and anti-mouse PE and FITC from Millipore. Flow cytometry analysis was performed on haemolysed whole blood or lymphocytes using BD FACSCanto or FACS Aria systems. For determining membrane and cytoplasmic staining, a DAKO Intrastain kit for flow cytometry was used (No. K2311; Dako). Mean fluorescence intensity for sortilin, trkA and p75NTR antibody staining on the membrane and inside the cell were obtained and analyzed using BD FACSDiva software (BD Biosciences). Data were represented as percentage (%) of mean fluorescence above control (secondary antibody). Results from separate experiments were pooled and represented as mean $\pm$ standard deviation (SD) and significance determined using Student T test (Prism 4; GraphPad Software, Inc).

2.2 Immunoblotting

Natural killer (NK) and total T cells were isolated from peripheral blood lymphocytes by cell sorting, gating on CD16/56 (CD3-negative) and CD 3 populations. CD3 negative CD16/56 positive populations were considered to be NK cells and CD3 positive cells were T cells. Sorted cells were immediately washed in PBS containing protease inhibitors (1.0 mM

EDTA, 1.0 µg/ml leupeptin, 1.0 µg/ml pepstatin A and 200 µM PMSF), subjected to low speed centrifugation (400 g), the pellets boiled in SDS buffer, and subjected to 4-12% SDS PAGE.

Natural Killer cells were also isolated from whole blood by negative selection using magnetic bead isolation (see below) and washed in PBS containing protease inhibitors. To generate whole cell lysates cells were lysed with a Retsch Tissue Lyser (setting 300, 3 minutes; Qiagen) in modified RIPA buffer (0.15 M NaCl, 1 mM EDTA, 10 mM Tris pH 7.2, 1% Triton X-100) containing Phosphatase inhibitors (PhosSTOP tablet; Roche diagnostics) and protease inhibitors (as above). Cell lysates were then centrifuged at 14,000 g to remove cell debris. Conditioned media collected from NK cells cultured in serum free media at 0, 20, 24 and 48 h with protease inhibitors added was spun at 2,000 g to remove cell debris and concentrated approximately 10-fold using Vivaspin4 5 kDa cut-off spin columns (Sartorius Inc). Protein concentration in all samples was estimated by the Biorad DC Protein Assay (Bio-Rad Laboratories).

For detection of sortilin and p75NTR, reducing conditions were employed with non-reducing conditions used for trkA detection as previously described (Kahle et al., 1994). Protein from PAGE gels were transferred to nitrocellulose and incubated in primary antibody using standard techniques. The monoclonal anti-human sortilin (extracellular residues 300-422) from BD biosciences was used to detect sortilin; polyclonal rabbit anti-p75NTR (intracellular domain 274-425; Millipore) and monoclonal anti-trkA (5C3, LeSauter et al., 1996) were used for p75NTR and trkA detection respectively. Rabbit anti-NRAGE and sheep anti-NGF were from Millipore. Bands were detected by secondary antibodies conjugated to alkaline phosphatase or horseradish peroxidase and developed with ECF or ECL substrates respectively (GE Biosciences; Pierce Thermo Scientific). Bands were visualized on either a GE 9400 Typhoon scanner or Fuji Imager (LAS 4000) system. Positive control protein samples were from SKNSH, a neuroblastoma cell line established by Biedler (kindly provided by Dr Bryone Kuss, Flinders Medical Centre, Australia). PC12 cells were from Dr Lloyd A. Greene (Columbia University, New York, NY), mNGF kindly donated by Prof. Ian Hendry (Australian National University, ACT, Australia) and human recombinant wild type proNGF from Alomone Labs. The Western blot loading control antibody, rabbit anti-β-actin was from Abcam.

2.3 NK Cell Isolation by Negative Selection

NK cell numbers in volunteer donors were determined using a panel of antibodies (CD3-FITC, CD16/56-PE, CD8-PECY7, CD4-APC, CD19-PECY5 and CD45APCCY7) and flow cytometry. The CD3 negative CD16/56 positive cells as a percentage of lymphocytes (gated by dual scatter) gave the percentage of NK cells in the lymphocyte population. Samples with greater than 12% of lymphocytes identified as NK cells were used for isolation. Briefly, peripheral blood lymphocytes (PBL) were obtained from 450 ml of blood using Lymphoprep. The NK cells were then isolated by negative selection with magnetic beads as described by the manufacturer (No.19055R Stem Cell Technologies; No. 130092657 Miltenyi Biotech). NK cell purity was checked using the same combination of antibodies mentioned above. NK cell populations were from 90 to 98% pure and around 2.5×10^7 cells were obtained from each separation. Although some cells were cryopreserved for RT-PCR, freshly isolated NK cells were used for all other experiments. Cells were immediately placed in 24-well plates in either AIM-V/ PSG serum free media or RPMI 1640/1% FBS/PSG (Invitrogen) in the presence or absence of human interleukin-2 or interleukin-12 (IL-2, IL-12; No. 158129 and 148129 eBiosciences). NGF receptor profiles of samples were then tested by flow cytometry.

2.4 Immunocytochemistry

NK cells isolated by negative selection were assessed for surface and intracellular expression of the sortilin, p75NTR and trkA receptors using immunocytochemical staining and confocal microscopy. NK cells were cultured in 24-well plates in AIM-V/ PSG serum free media and receptor profiles of samples examined at 0, 24 and 48h. In short, 8×10^5 cells were washed twice in PBS, fixed for 10 minutes in 4% paraformaldehyde and again washed twice in PBS. In experiments that aimed at detecting intracellular expression of the two receptors, cells were permeabilized for 30 minutes in PBS-BSA (3%)-TritonX-100 (0.2%) at room temperature. The cells were then incubated with the primary antibodies overnight at 4° C. In experiments aiming at detecting surface expression of the receptors, the cells were incubated overnight at 4° C with primary antibodies diluted in PBS-BSA without prior permeabilization. Primary antibodies used were rabbit anti-sortilin to the whole extracellular domain (1/400; (Munck Petersen et al., 1999), anti-trkA 5C3 (10 µg/ml; (LeSauter et al., 1996) and anti-p75NTR MLR2 (20 µg/ml; (Rogers et al., 2006). Cells were then washed twice and incubated with Alexa-fluor 488 anti-rabbit or 647 anti-mouse (1/250) for 45 min at room temperature (Invitrogen). After a further two washes cells were mounted on microscopic slides using Slow Fade Gold mounting media (Invitrogen) and viewed under a confocal microscope (Leica TCS SP5, Leica Microsystems). Negative controls were incubated with primary or secondary antibodies alone to correct for auto fluorescence and non-specific binding.

2.5 Apoptosis/ Cell death assay

NK cells freshly isolated by negative selection were treated with cleavage resistant proNGF123 (Pagadala et al., 2006) or mNGF for periods up till 48 h. This was also done in the presence of human IL-2 or IL-12 (10 ng/ml; (Ross and Caligiuri, 1997). Full-length neurotensin (No. N6383 Sigma-Aldrich) was added to cultures before proNGF or NGF treatment. Methods used to detect apoptosis by flow cytometry were as described by the manufacturer using Annexin V-Alexa Fluor 488 and Propidium Iodide (Millipore Corp; Invitrogen). A total of 50,000 events were analyzed by multiparameter flow cytometry immediately after staining. Data was analyzed using BD FACSDiva software (BD Biosciences). PI negative/ Annexin V-Alexa Fluor 488 negative were assumed alive; PI negative/ Annexin V-Alexa Fluor 488 positive apoptotic; and PI positive/ Annexin V-Alexa Fluor 488 positive dead (or late apoptotic). The percentage of alive, apoptotic and dead cells were determined for treatments and controls as a % of the total amount of cells analyzed. Results from separate NK cell samples were pooled and represented as mean values $\pm$ standard deviation (SD). Significance was determined using ANOVA and posthoc testing (Bonferroni's Multiple Comparison Test). P values below 0.05 were regarded as significant (Prism 4; GraphPad Software, Inc).

2.6 RT-PCR

The expression of sortilin (NTR3), trkA, p75NTR (TNFRSF16; NGFR) and β -actin in sorted NK cells were examined by RT-PCR. The primer sequences and expected product sizes are given in Table I. The Illustra Quickprep Micro mRNA purification kit (GE Health) was used to prepare mRNA from sorted NK cells, and cDNA was generated using Superscript III (Invitrogen). PCR amplification was performed with an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 10min.

3. Results

3.1 Distribution of NGF receptors in blood cells

The expression of sortilin in peripheral blood lymphocytes (PBL) was examined in lymphocyte subsets using multi-parameter flow cytometry that included markers for T, B and NK cells. Lymphocytes were gated on CD45 and SSC expression. These cells were then examined for co-expression of a number of antigens. T cells were identified on CD3 expression. NK cells were identified as CD3 negative, CD16/CD56 positive. B cells were identified as being CD3 negative and CD19 positive. Expression of Sortilin, p75NTR or trkA expression was determined on these different populations. Fig. 1 represents data from a blood sample of one of eight individuals examined. Surprisingly, sortilin was detected on the cell surface of the majority of NK cells in the lymphocyte population (Fig. 1 **B2**). In addition, sortilin stained 50% of B cells (Fig. 1 **B6**) but was rarely detected on T cells (Fig. 1 **B10**). Sortilin antibody stained 80.6 ± 2.5 % (mean $\pm$ SD; n=8) of all the CD56-positive NK cell population.

PBL were also examined for expression of the NGF receptors p75NTR and trkA as above. Fig. 2 is from one of eight samples. TrkA using the monoclonal antibody 165126 was found on nearly half of all NK cell (Fig. 2 **B2**) but was rare on B cells and appeared absent from T cells (Fig. 2 **B6, B10**). Flow cytometry data from all individuals indicated that trkA was detected on 45 ± 2.1 % of NK cells (mean $\pm$ SD; n=8). P75NTR cell surface staining of NK and B cells using the MLR2 monoclonal antibody was negligible (Fig. 2 **C2, C6**) and was absent from T cells (Fig. 2 **C10**). To confirm that p75NTR is absent on the surface of lymphocytes, several other monoclonal antibodies were also used. All antibodies (ME20.4, 8211, and NGFR5 clones; (Marano et al., 1987; Ross et al., 1984)) gave the same negative surface binding on NK, T and B cells (data not shown).

3.2 Expression of NGF receptors by NK cells

To further examine the expression of NGF receptors and the co-receptor for proNGF, sortilin, Western blots were used to detect proNGF and NGF receptors in NK cells isolated from human PBL. This method can detect the presence of receptors in the entire cell, whereas the previous flow cytometry (Fig. 1 and 2) was used to detect receptors on the surface of cells. Cell sorting of PBL with CD16/56 and CD3 was used to differentiate NK from T cells. Fig. 3 shows Western blots of NK and T cell pools using this positive selection method. The 100kDa band was detected with sortilin antibody in the NK cell but not T cell pools (Fig. 3). The expected 140-kDa band was seen for trkA in NK cells as well as the 110-kDa band previously described in other human tissue as the trkA precursor (Kahle et al., 1994). A small amount of trkA was detected in T cells. Surprisingly, p75NTR was also detected in NK cells and at low amounts in T cells.

We then examined whether p75NTR and sortilin were expressed intracellularly and extracellularly in NK cells by both flow cytometry and confocal imaging. Further examination of freshly isolated NK cells using flow cytometry revealed there was no difference between surface and cytoplasmic sortilin staining in NK cells, suggesting that sortilin is mainly on the membrane. However, intracellular p75NTR and trkA were at significantly greater levels than that found on the surface of NK cells (Fig. 4A). We next examined membrane and cytoplasmic expression of P75NTR, trkA and sortilin by confocal imaging after 48 h hours of culture in serum free media. Fig. 4B shows sortilin staining is similar on non-permeabilized and permeabilized cells. Membrane and cytoplasmic expression of p75NTR appears after 48 hours in serum free conditions (Fig. 4B). TrkA was detected on the surface and significant amounts found inside the cell (Fig. 4B).

To confirm the possibility that NK cells have receptors for proNGF, sortilin, trkA and p75NTR, expression was examined by RT-PCR in primary un-stimulated NK cells (Fig. 4C). P75NTR mRNA was detectable at low level. Sortilin mRNA was detected in primary NK cells. The PCR products for trkA agree with previous published results published using the same primers (Nassenstein et al., 2006).

3.3 The p75NTR receptor is functional in NK cells

Initially, we investigated if p75NTR is capable of inducing second messenger proteins associated with apoptosis in NK cells. One of the second messengers, neurotrophin-receptor-interacting-MAGE-homologue (NRAGE), is up regulated on NGF binding to p75NTR (Salehi et al., 2000). NK cells isolated by negative selection were treated for 30 minutes with 100 nM proNGF123 or mNGF followed immediately by isolating whole cell lysates. Western blots using an antibody known to detect NRAGE (Salehi et al., 2000) showed that after 30 minutes treatment with 100 nM proNGF123, an 88kDa band was present that was not apparent in untreated NK cells (Fig. 5). At the same concentration, NGF also induced NRAGE expression, but at lower levels. As a control for second messenger activation, PC12 (a neuronal cell-line known to up-regulate NRAGE following NGF treatment) cells were tested. When PC12 cells were treated with proNGF and NGF the 88kDa band appeared but was absent in untreated cells (Fig. 5).

3.4 ProNGF induces apoptosis in NK cells

We next sought to determine if proNGF could induce death of NK cells by binding the sortilin-p75NTR complex. Since the presence of serum is known to complicate interpretation of NGF (and proNGF) action we used serum-free conditions to assess the affect of these factors on primary NK cells. Secondly, cleavage-resistant proNGF was used to prevent the affect of NGF being cleaved from its pro-form during cell culture. This mutated proNGF123 form has been previously shown to be biologically active (Pagadala et al., 2006).

ProNGF123 was found to induce cell death in unstimulated NK cells and in NK cells cultured in the presence of IL-12 (Fig. 6). Cell death was assessed by Annexin V-Alexa Fluor 488 / PI binding and flow cytometry. Cleavage resistant proNGF123 and mature NGF were tested at a range of concentrations; the most consistent results being seen with 10 to 100 nM proNGF123 (0.64 to 6.4 ng/ml) and 100 to 1000 nM mature NGF (2.6 to 26 ng/ml).

After 20 hours of culture, a small but significant amount of apoptosis was observed for NK cells treated with 100 nM proNGF123 in the presence of IL-12 compared to 100 nM proNGF123 alone (Fig. 6A; $p < 0.01$; $n = 4$). Blocking the proNGF co-receptor sortilin with the sortilin ligand neurotensin (20 μ M), reduced apoptosis caused by proNGF123 in the presence of IL-12 back to that observed with IL-12 alone ($p < 0.05$; $n = 4$). This suggested that the presence of IL-12 may be increasing p75NTR expression. When proNGF and NGF receptor expression were examined by flow cytometry, there was a significant (10 fold) increase in p75NTR receptor levels compared to cultures not containing IL-12 (Fig. 6B). Surprisingly, this also occurred with IL-2, even though no apoptosis was observed (data not shown). There was no change in sortilin or trkA expression.

Cell death induced by proNGF123 and NGF was not seen without the presence of IL-12 at 20 h treatment. However, after 48 h, dead (or late apoptotic) NK cells were also observed after exposure to proNGF123 (Fig. 6C). The affect of proNGF123 was blocked by neurotensin ($p < 0.001$; $n = 4$) and was comparable to the cell death induced by IL-12 alone. There was a small increase in cell death induced by proNGF123 in the presence of IL-12,

some of which was also blocked by neurotensin (Fig. 6C; $p < 0.01$; $n = 4$). Mature NGF appeared to have no effect at 48 hours.

The sortilin ligand neurotensin did not improve the survival of NK cells when cultured in the presence or absence of IL-12 or 2 either at 20 or 48h, suggesting these cells do not constitutively produce proNGF (Fig. 6A and C). This was examined by culturing primary NK cells in serum free media without treatment, and subjecting concentrated conditioned media to Western blot with an antibody that recognizes both pro and mature NGF (Fahnestock, 2001). There was no proNGF (or mature NGF) detected in conditioned media collected at 0, 24 and 48 h after NK cells were cultured in serum-free media or media collected from cells cultured in IL-12 (10 ng/ml) for 20 h (results not shown).

4. Discussion

Until now, the biology of proNGF binding to the co-receptors p75NTR-sortilin has been investigated only in the nervous system. We now identify p75NTR and sortilin on NK cells and show that these receptors respond to proNGF by inducing NK cell death. Our results suggest that in the presence of IL-12, p75NTR is up-regulated and this leads to an increased apoptotic effect of proNGF123 or NGF. Cytokine priming (by, for example, IL-12) renders NK cells susceptible to AICD, the kinetics of which are very slow (Ross and Caligiuri, 1997).

Our flow cytometry data of whole blood samples showing trkA on NK cells is in agreement with the only published study showing this NGF receptor may be on NK cells (Nassenstein et al., 2006). NK cells appear to contain large amounts of intracellular trkA by flow cytometry, confocal imaging and Western blot of whole cell lysates. We demonstrate that sortilin is expressed mainly as a membrane receptor on NK cells. The receptor appears to be on the surface of primary NK cells isolated from blood. Confocal imaging supports this data. Western blotting show the appearance of the 100-kDa sortilin band in NK cells. Previous research has revealed that intracellular sortilin is smaller (around 90 kDa) than membrane bound sortilin (Morinville et al., 2004; Navarro et al., 2001). Indeed the predominance of sortilin on the cell surface in NK cells is unusual as sortilin expressed in neurons, adipocytes and transfected CHO cells is present (over 90%) inside the cell with only minor amounts being on the cell surface (Morinville et al., 2004; Morris et al., 1998; Petersen et al., 1997). This may suggest that sortilin is not involved in cytoplasmic trafficking or sorting in NK cells. Our data showing that neurotensin does not prevent cell death suggests that NK cells may not constitutively produce proNGF. Indeed when we examined concentrated conditioned media from untreated NK cells cultured either for 20h with IL-12, or over 48 hours in serum free conditions, no proNGF was detectable by Western blot. Taken together sortilin appears to be a significant cell surface co-receptor for proNGF but not for cytoplasmic trafficking of proNGF produced by NK cells. This contrasts with B cells where recent data shows that secretion of BDNF is regulated by sortilin (Fauchais et al., 2008). Blocking the sortilin receptor down-regulates BDNF in an autocrine manner, where cytoplasmic trafficking of proBDNF and secretion of the mature BDNF are dependent on sortilin (Fauchais et al., 2008). In addition, in contrast to NK cells, there appears to be very little membrane expression of sortilin on B cells. Our data suggest that, unlike in other immune and neuronal cells, sortilin does not regulate intracellular trafficking and autocrine production of neurotrophins in NK cells. The fact that NGF transcripts were not previously found in a study that looked at TNF ligands in NK cells supports this (Kashii et al., 1999).

Freshly isolated NK cells were found to contain cytoplasmic rather than cell surface p75NTR. This cytoplasmic location is unusual but similar to what has been found recently in B cells (Fauchais et al., 2008). P75NTR was present in all B cell lines, with an

intracytoplasmic sequestration in standard cultures (10% FCS). Fauchais (Fauchais et al., 2008) showed membrane relocation after a day of serum-free culture and concluded this was a stress response.

There was a low basal level of cytoplasmic p75NTR in NK cells. p75NTR is present at low levels in many neuronal cell types until signalling from ligand binding or cell stress increase expression and insertion of the receptor into the membrane (Blochl and Blochl, 2007; Bronfman et al., 2007; Kobayashi et al., 2002 for review). Our data demonstrates that p75NTR receptor expression is up regulated intracellularly in NK cells after 20 h culture in the presence of IL-12 or IL-2. This is in agreement with the interleukins being “stress” signals that increase p75NTR cytoplasmic receptor expression before insertion into the membrane upon exposure to NGF or proNGF (Bronfman et al., 2007; Bronfman et al., 2003). IL-2 has previously been shown to up-regulate p75NTR expression in B cells and IL-2 activated B cells have been shown to undergo proliferation in the presence of NGF (Brodie and Gelfand, 1992). However, further research determined that it was NGF binding to trkA that initiates this proliferative response in B cells following IL-2 treatment (Melamed et al., 1996). The apoptotic affect of NGF or proNGF had not been investigated in B cells. IFN- γ , TNF- α and TNFR are up regulated in both IL-2 and IL-12 treated NK cells (Naume et al., 1992; Ross and Caligiuri, 1997). A previous report shows that TNF- α induces up-regulation of trkA but not p75NTR in activated macrophages (Barouch et al., 2001). The data we present in this paper indicates that trkA is not up-regulated in the presence of either IL-2 or IL-12.

ProNGF123 caused NK cell death after 48h in serum-free medium without IL-12. This may be the result of p75NTR function changing with time as demonstrated by a study showing primary neuronal apoptosis increases with age (Brann et al., 2002). However, our data shows that similar to B cells (Fauchais et al., 2008), cell surface p75NTR expression in NK cells increases in control untreated cells after culture in serum free media. This again may be the “stress response” to low serum conditions described by Fauchais et al. (Fauchais et al., 2008) resulting in up-regulation of p75NTR receptor expression that makes available the required level of receptors for apoptosis.

NK cell apoptosis occurred after 20h culture in the presence of IL-12. The mechanism appears to involve up-regulation of p75NTR. Blocking sortilin, diminished proNGF123 but not mature NGF-induced cell death back to that seen with IL-12 alone, indicating that proNGF requires sortilin to induce cell death. The apoptotic affect seen with IL-12 (but not IL-2) in untreated NK cells has previously been observed (Ross and Caligiuri, 1997) and associated with AICD.

After a 30-minute exposure to NGF or proNGF123, NRAGE was detected. This indicates both the non-cleaved and mature forms of NGF induce apoptosis through the p75NTR receptor and is in agreement with data showing that the mature domain of proNGF induces intracellular signals that conclude with cell death (Nykjaer et al., 2004). P75NTR mediated apoptosis requires activation of Jun kinase, phosphorylation of BH3-domain only family members, release of mitochondrial contents and activation of caspase-9 (Blochl and Blochl, 2007). Independent studies have shown that several members of the MAGE protein family bind to the cytosolic region and one of them, termed NRAGE (also known as Maged1 and Dixon) is an indicator that apoptosis can occur. This second messenger is present when ligand binding to p75NTR signals independently of trkA in apoptosis (Bhakar et al., 2003; Salehi et al., 2000). In agreement with our results, NRAGE is recruited to p75NTR in a neurotrophin dependent manner. Our results therefore indicate that p75NTR-mediated apoptosis can occur in immune-system cells.

NRAGE was detected soon after exposure to proNGF or NGF, but cell death in the presence of IL-12 occurs 20h later or 48h later in the absence of IL-12. This is in agreement with previous research (Salehi et al., 2000). The reason for this delay is due to ligand-induced up-regulation of p75NTR in concert with recycling of the receptor-ligand through the endosome (Bronfman et al., 2007; Bronfman et al., 2003). Binding of NGF or proNGF to p75NTR increases membrane p75NTR and this in turn increases apoptosis (Bronfman et al., 2007; Bronfman et al., 2003). Although IL-12 induces p75NTR after 20h, continued insertion of p75NTR into the membrane requires ligand binding. Our data also shows that the apoptotic affect of NGF and proNGF in unstimulated NK cells is not observed until after 48h exposure. Presumably, the up-regulation of p75NTR on the surface of NK cells by IL-12 speeds up this affect.

There is currently no information on proNGF action in NK cells. There is a possibility that proNGF/NGF may also have a role in autoimmune diseases of the central nervous system such as multiple sclerosis (MS) where NK cell depletion is well established (Gandhi et al., 2009). We show that proNGF is functional as NK cells die in the presence of proNGF. As part of normal homeostatic mechanisms, NK cells can die through the action of interleukins and TNFR ligands. Ross and Caligiuri (1997) showed that priming (by, for example, IL-12) can render NK cells susceptible to AICD and suggested this may occur *in-vivo* after sustained infectious insults when other more reversible mechanisms of negative feedback are inoperative or ineffective. Where would NK cells encounter proNGF *in-vivo*? ProNGF produced by other cells could be involved in this AICD. However, it is unlikely proNGF could be part of Fas induced NK cell suicide on tumor cell engagement because NK cells do not produce proNGF. Rather, similar to GITRL, proNGF/ NGF must be produced in the microenvironment surrounding NK cells. In the immune system activated macrophages, mast cells, basophils, eosinophils, and activated B and T cells all produce NGF in allergic or inflammatory conditions (Aloe et al., 1999). Whether proNGF/NGF released by for example monocytes after acute infections can kill cytokine primed NK cells is not known at present. Validation of these hypotheses *in-vivo* could be clearly assessed with the help of transgenic and knockout animal models. For example, proNGF killing of NK cells could be assessed in sortilin knockout animals (Jansen et al., 2007) when they become readily accessible.

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Figure 1. Sortilin stains natural killer (NK) cells and some B cells

Flow cytometry analysis of peripheral blood lymphocytes isolated from normal human blood stained with NK cell (CD56PECY5), B cell (CD19PECY7) and T cell (CD3PE) markers and sortilin (rabbit anti-sortilin). 14% of the total lymphocyte population were NK cells and 83% of these stained with the sortilin antibody (**B2**). B cells comprised 6% of the lymphocyte population and 49% of these cells were positive for sortilin. T cell staining with anti-sortilin was less than 1% of total T cells and there was minimal secondary staining with anti rabbit-FITC (**A2**, **A5** and **A10**). The lymphocyte population was gated from C45APCCY7 and side scatter plot (data not shown). This is flow cytometry analysis from one normal human donor; eight were analyzed.

Figure 2. TrkA is present on a small population of NK cells and some B cells, whereas p75NTR does not appear to be present on any lymphocytes

Flow cytometry analysis of peripheral blood lymphocytes isolated from normal human blood stained with NK cell (CD56PECY5), B cell (CD19PECY7) and T cell markers (CD3APCCY7) in comparison to NGF receptor antibodies, monoclonal anti-trkA (graphs with **B** quadrants); and anti-p75NTR (graphs with **C** quadrants). TrkA antibody stained 56% of NK cells (**B2**) and 16% of B cells (**B6**) and no T cells. P75NTR staining of NK, B and T cells was similar to control anti-mouse PE (**C2**, **B6**, **C6**). Secondary staining with anti-mouse PE is shown in graphs with **A** quadrants. (Representative flow analysis from a normal human donor; eight normal donors were analysed).

Figure 3. Expression of sortilin, p75NTR and trkA by NK cells

One million NK and T cells sorted from normal PBL cells by positive selection were boiled in SDS PAGE buffer and subject to Western blot. Sortilin and trkA were detected in NK but not T cells. A small amount of P75NTR was found in NK cells and trace amounts in T cells. A membrane fraction (20 μ g) from a human neuroblastoma cell line, (SKNSH) was included as positive control for p75NTR and sortilin. PC12 whole cell lysate (20 μ g) was used as a positive control for trkA. The loading control antibody for β -actin was also included. This is one of four experiments with sorted samples from PBL.

Figure 4. Sortilin, p75NTR and trkA expression in unstimulated NK cells

Freshly isolated NK cells were labelled with sortilin (rabbit anti-sortilin, 1/500), or trkA (mouse anti-trkA 5C3, 20 μ g/ml) or p75NTR (mouse anti-p75NTR, 50 μ g/ml) with and without permeabilization to detect cell surface and intracellular antigens, the cells were subject to flow cytometry (**A**). There was membrane expression of sortilin with no evidence of cytoplasmic staining. A significant amount of p75NTR and trkA was detected in the cytoplasm compared to the cell surface (** $P < 0.05$). Results are expressed as mean fluorescence intensity above control secondary anti-rabbit 488 (sortilin) or anti-mouse PE (trkA and p75NTR) from six separate samples (mean and standard deviation $SM \pm SD$). Confocal microscopy was performed on unstimulated NK cells maintained for 48 hours in serum-free conditions (**B**). Representative micrographs show sortilin, p75NTR and trkA staining were present on the cell surface on non-permeabilized cells after 48 hours in culture, but sortilin was mainly localised to the plasma membrane in permeabilized cells,

while there was abundant cytosolic immunoreactivity for p75NTR and trkA. No p75NTR was detected on the cell surface of freshly isolated NK cells before 24 hours in culture (data not shown). Scale bars = 10 μ m. RT-PCR analysis was performed to further confirm the expression of sortilin, p75NTR and trkA in NK cells (C). Transcripts for sortilin (552 (a), 199 bp (b)), trkA (479 and lower 400 bp) and p75NTR (200 bp) were detected, β -actin (838 bp) is included as control for RT-PCR. This are results from one of four separate experiments. Data were analysed by student T-tests (Prism4; Graph Pad software).

Figure 5. Second messenger for p75NTR-induced apoptosis is detected in NK cells treated with ProNGF123 and NGF

NK cells isolated by negative selection were cultured in AIM-V medium with and without cleavage resistant proNGF123 100 nM (6.4 ng/ ml), and mature NGF (100 nM; 2.6 ng/ ml). After 30 minutes, cells were processed for Western blot and 20 µg of each sample subject to SDS PAGE and then rabbit anti-NRAGE and the blot developed with anti-rabbit HRP and ECL. Lysates from PC12 cells treated with and without proNGF123 and NGF (100 nM) were included as controls. The blot was also subject to anti-β-actin as a loading control.

Figure 6. ProNGF123 induces death of NK cells in the presence of IL-12 after 20h or in unstimulated NK cells after 48h serum free culture

NK cells isolated by negative selection were cultured in AIM-V medium with and without IL-2 or IL-12 (10 ng/ml) and either cleavage resistant proNGF123 (100 nM; 6.4 ng/ml), or mature NGF (100 nM; 2.6 ng/ml) in addition to neurotensin (20 μ M). Cells that were Annexin positive/ PI negative were classified apoptotic. After 20 hours (A) there were significantly ($**$; $p < 0.01$) more apoptotic NK cells after proNGF123 or NGF treatment in the presence of IL-12, compared to treatments without IL-12. Neurotensin (NT) reduced the apoptotic effect of proNGF123 but not mature NGF cultured in IL-12, back to that seen with IL-12 alone. IL-2 appeared to promote cell survival in all cultures. Results are presented as

mean and standard deviation from experiments on four separate NK isolates (mean $\pm$ SD). NK cells cultured with and without IL-12 (10 ng/ ml) for 20 h were also examined by flow cytometry (**B**) for membrane and intracellular sortilin, p75NTR and trkA antigens as shown in Fig 4A. In the presence of IL-12, intracellular p75NTR staining increased 10 fold. There appeared to be little change in sortilin or trkA expression in the presence of IL-12.

ProNGF123 promotes death in un-stimulated NK cells after 48h (C). NK cells were isolated by negative selection and cultured with and without cleavage resistant proNGF123, or NGF in the presence and absence of IL-12 in addition to the sortilin blocker neurotensin (NT) as in (**A**), but for 48h. Cells that were Annexin positive/ PI positive were classified dead, and % of dead cells is indicated. After 48h, there were significantly ($p < 0.001$) more dead NK cells (**) after proNGF123 or IL-12 treatment. The effect of proNGF123 was blocked by neurotensin ($p < 0.001$) and was comparable to the cell death induced by IL-12 alone. There was a small increase in cell death induced by proNGF123 in the presence of IL-12, some of which was also blocked by neurotensin ($p < 0.01$). Mature NGF appeared to have no effect. Results are presented as mean and standard deviation from experiments on four separate NK isolates (mean $\pm$ SD). Data were analyzed by one-way ANOVA and then posthoc Bonferroni's Multiple Comparison Test (Prism 4; Graphpad software).

Table I
Primers used in RT-PCR of NK cells

Target	Primer Sequences	Expected Product	Reference
Sortilin a	sortilin-F: 5'-AGAATGGTCGAGACTATGTG-3' sortilin-R: 5'-AAGAGCTATTCCAAGAGGTCC-3'	552bp	Martin et al., 2003
Sortilin b	sortilin-F: 5'-CTGGGTTTGGCACAATCTTT-3' sortilin-R: 5'-CACCTTCCTCCTTGGTCAAA-3'	199bp	Fauchais et al., 2008
p75NTR (NGFR)	p75NTR-F: 5'-GTGGGACAGAGTCTGGGTGT-3' p75NTR-R: 5'-AAGGAGGGGAGGTGATAGGA-3'	200bp	Fauchais et al., 2008
TrkA	TrkA-F: 5'-AATGCCTCGGTGGATGTG-3' TrkA-R: 5'-AGCGTGTAAGTTGCCGTTGTT-3'	479bp	Nassenstein et al., 2006
β -actin	β -actin-F: 5'-ATCTGGCACCACACCTTCTACAATGAG CTGCG β -actin-R: 5'-CGTCATACTCCTGCTTGCTGATCCACATCT GC	838bp	Jutel et al., 2001

F, = forward; R, = reverse