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Pro Nerve Growth Factor and its receptor p75NTR activate inflammatory responses in synovial fibroblasts: a novel targetable mechanism in arthritis.

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1. Introduction:

1.1 Nerve Growth Factor and the other neurotrophins

Neurotrophic factors play a central role in nervous system development, covering a broad range of effects: from the regulation of neuronal cell death and differentiation during embryo development to the control of neurite outgrowth and peptide synthesis in adult life. The acknowledged prototypical example and best-characterized neurotrophic factor is nerve growth factor (NGF) [1]

discovered by Rita Levi-Montalcini in the 1950s. The pioneering studies of Levi-Montalcini showed that, during chick embryo development, limited amounts of NGF produced in target organs are responsible for the proliferation, differentiation and survival of peripheral autonomic and sensory neurons [1]. Moreover, the neutralization of endogenous NGF with anti-NGF antibodies during fetal life causes death of sympathetic neurons and a marked decrease in sensory neurons[1]. In adult life, while sympathetic neurons continue to depend on NGF for their survival there is a change in the effects of NGF on sensory neurons. NGF is no longer essential for their survival but remains crucial for the maintenance of their differentiated phenotype [2] [3]. In these mature sensory neurons, as well as in sympathetic neurons, NGF dynamically controls neurotransmitter and neuropeptide synthesis. In sympathetic neurons the levels of norepinephrine are regulated by NGF through the selective induction of tyrosine hydroxylase (TH) [4]. In dorsal root ganglia (DRG) the expression of neuropeptides such as Substance P (SP) and Calcitonin Gene-Related Peptide (CGRP) in primary sensory neurons is under NGF control [5] and *in vivo* deprivation of NGF, following nerve transection or anti-NGF treatment, causes a marked decrease in SP and CGRP synthesis [3]. In addition to its functional activity in sympathetic and neural crest-derived sensory neurons, NGF has a key function in the central nervous system (CNS) regulating for development and maintenance of basal forebrain cholinergic neurons [6]

Tissue sources of NGF in the periphery are typically non-neuronal cells, whereas in the CNS NGF is for the most part synthesized by neurons—at least under physiological conditions. The production of NGF in the embryonic peripheral tissues influences the growth of axons and dendrites, enabling neurons to establish connections with their targets and ensuring proper neural network formation. In the adults, the NGF supplied by the innervation field influences neuronal plasticity allowing nervous system to modify its structure and functions in response to stimuli. Indeed, the constitutive synthesis of NGF in adult tissues controlls the phenotypic features of PNS neurons, such as innervation density, cell body size, axonal terminal sprouting, dendritic arborization, induction or inhibition of neuropeptide and neurotransmitter synthesis or transmitter-producing enzymes [4] [5] [7] [3]. Moreover, in damaged peripheral nerves NGF plays a key role in nerve regeneration after injury by promoting axon growth and the restoration of neuronal connections. In the CNS, NGF is involved in modulating the ability of synapses to strengthen or weaken over time (synaptic plasticity) a key process crucial for learning and memory processes.

Almost forty years after NGF discovery, a second neurotrophic factor, brain-derived neurotrophic factor (BDNF) was isolated [8] [9]. BDNF shares a great structural similarity with NGF, leading to the concept of the neurotrophin family. Later studies identified two additional members of this family: NT3 (neurotrophin 3) and NT4 (neurotrophin 4) [10] **(Figure 1)**.

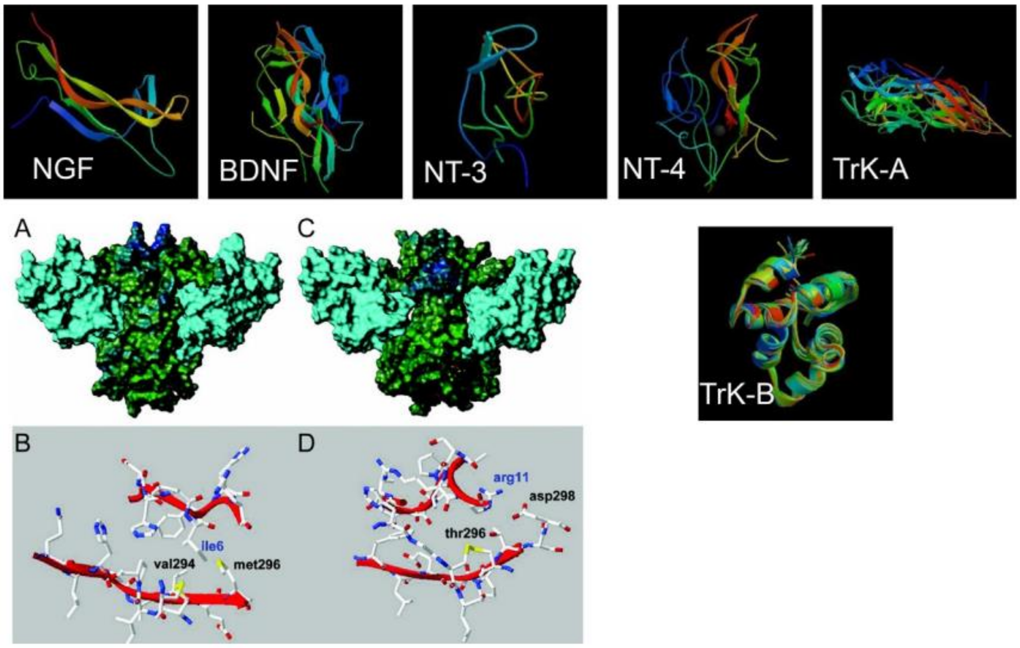

Figure 1. Structure of the neurotrophins. The figure shows the 3D structure of each identified neurotrophins and the X-ray crystallographic structures of NGF-TrkAD5 (A) and NT-4-TrkBD5 (C), and interactions between the N-terminus of the neurotrophin with the D5 domain of the receptors (B and D). [11]

These neurotrophins have been found in all vertebrates, while two additional neurotrophins closely related to NGF, neurotrophin-6, and neurotrophin-7, have been found only in fish [12] [13] [14]. All neurotrophins share structural similarities and analogous functional roles in the nervous system, regulating neuronal survival, growth, differentiation, and synaptic plasticity. The study of neuro-trophin structure showed that they are homodimeric proteins; each subunit consists of about 120 amino acids, forming a “cysteine-knot” motif [15], which has three disulfide bridges that maintain its three-dimensional shape, making the protein resistant to proteolytic degradation.

1.2 proNGF processing

All neurotrophins are initially synthesized as proneurotrophin precursors whose enzymatic cleavage yields to mature neurotrophins The functional NGF protein is derived from its precursor, proNGF, which is synthesized in neurons and secreted into the extracellular space where undergoes proteolytic cleavage and becomes the mature, bioactive NGF.[16]. The domain of proNGF is 241 aa in length and has a molecular mass of ≈32 kDa, is synthesized in the endoplasmic reticulum (ER) and subsequently processed into the Golgi apparatus. Proteases such as furin, matrix metalloproteinases (MMPs) (e.g., MMP-7), and plasmin can cleave proNGF removing the prodomain. This processing typically occurs at the Lysine-Arginine (KR) site, located at the boundary between the prodomain and the mature NGF sequence. The mature NGF is a non-covalently linked homodimer, constituted by subunits of 118 amino acids, with a molecular weight of approximately 13 kDa per monomer. The discovery that proneurotrophins are biologically active [17] has revolutionized the field of neurotrophin research. ProNGF appears to have biological effects [17] that are distinct from, or even opposite, to those of mature NGF in certain cell types [18]. *In vivo* studies have shown that proNGF and not the mature NGF is the prevalent form present in the brain [19] and peripheral nervous tissues [20]. Levels of proNGF in the brain increases during aging [21], in neurodegenerative diseases (i.e., Alzheimer's and Parkinson's diseases) [19];and diabetic encephalopathy [22].

1.3 NGF receptor system

The diverse functions of the neurotrophins are mediated *via* two classes of receptor: the Trk (tropomyosin receptor kinase) family of RTKs (receptor tyrosine kinases) and p75NTR (p75 neurotrophin receptor), a molecule belonging to the large family of TNF-receptor homologs. The molecular interplay between Trks and p75NTR receptors is complex and incompletely understood but it triggers cellular effects that are highly ligand and cell type specific.

1.3.1 TrkA receptor

The Trk family has three members, namely TrkA, TrkB, and TrkC. Each of these receptors has a preferred neurotrophin ligand: NGF binds preferentially to TrkA, BDNF and NT-4/5 bind to TrkB, and NT-3 binds to TrkC with binding affinities (Kd) of 10^{-9} –10^{-10} M [23] [24]. The expression and functional activity of each Trk member in the nervous system is cell-type specific (albeit with some crossover and co-expression). For example, TrkB and TrkC are highly expressed in cortical and hippocampal pyramidal neurons and cochlear neurons, whereas TrkA is found in cholinergic basal forebrain neurons. TrkA is encoded by the NTRK1 gene located on chromosome 1q21-q22 [25].

TrkA is a transmembrane tyrosine kinase receptor, constituted by an extracellular domain, a transmembrane region and an intracellular region containing the tyrosine kinase domain. The extracellular domain contains a cysteine-rich cluster (C1) followed by three leucine-rich 24-residue repeats (LRR1–3), another cysteine-rich cluster (C2) and two immunoglobulin-like domains (Ig1 and Ig2) (**Figure 2A**). The intracellular region contains five key tyrosine residues: three within the activation loop of the kinase domain, which are necessary for full kinase activity, and two on either side of the tyrosine kinase domain, which serve as phosphorylation dependent docking sites. TrkA signalling pathway is initiated when NGF binds to TrkA receptors at the cell surface causing the formation of receptor dimers. The dimerised receptor auto phosphorylates specific tyrosine residues in the activation loop of the kinase domain [Y676, Y680 and Y681 in TrkA (Figure 2B). This phosphorylation is required for activation of the Trk receptor [26] and leads to subsequent phosphorylation of additional tyrosine residues (Y496 and Y79), enabling docking of cytoplasmic adaptors and enzymes [27] and activation of a variety of downstream signalling pathways. The NGF binding to TrkA causes activation of the RAS/MAPK pathway, leading to increased cellular proliferation and growth via ERK signaling [28] [29] [30] [31]. The MAPKs activated by TrkA as

ERK1/ERK2, and ERK5 phosphorylate and activate the downstream transcription factors CREB, Elk-1, and MEF2, to regulate target gene expression that contributes to neuronal differentiation and survival [32] [33]. Another pathway activated by TrkA is PI3K that plays an important role in cell survival by inducing Akt activation, phosphorylation and inhibition of apoptosis-promoting proteins such as Bad and GSK3β [34]. PLC-γ1 phosphorylation and activation by TrkA leads to production of IP3 and DAG, which in turn promote Ca2+ mobilization and activation of a number of protein kinase C isoforms [35]. The PLC-γ1 activation is sustained and induces target gene expression such as the PN1 sodium channel gene [36] **(Figure 2B)**.

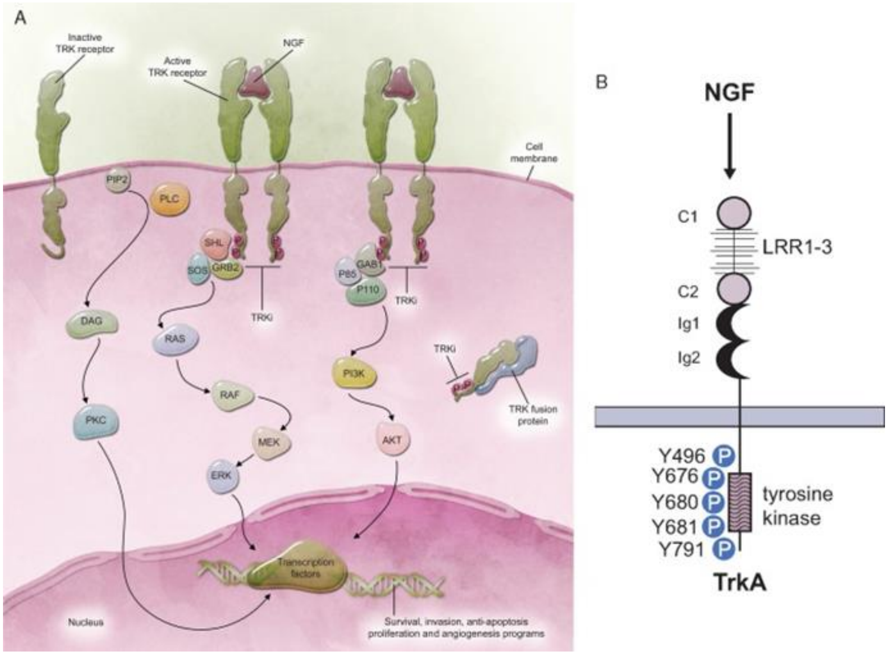

Figure 2. Signal-transduction pathway of the TrkA tyrosine kinase receptor. (A) Overview of TRK signalling pathway; (B) activation of TRKA. C1/C2, cysteine-rich clusters; Ig1/Ig2, immunoglobulin-like domains; LRR1–3, leucine-rich repeats; NGF, nerve growth factor; TRK, tropomyosin receptor kinase, TRKi, tropomyosin receptor kinase inhibitor.[37]

TrkA receptors are predominantly expressed in neuronal tissue and play an essential role during embryonic development as well as in the normal function of the nervous system [27].[38]. The activation of TrkA by NGF has an impact on a variety of neuronal events, such as neuronal cell differentiation and survival, cell proliferation, synaptic formation and plasticity, membrane trafficking, and axon and dendrite formation [39] [40]. TrkA receptor and NGF have been implicated in the survival and differentiation of sensory ganglia and are expressed in almost all nociceptive neurons in the dorsal root and trigeminal ganglia [41] [42].

The majority of studies on TrkA signaling have been performed using either a cellular model of mouse pheochromocytoma, the PC12 cell line [43], which in the presence of NGF differentiates in sympathetic neurons, or primary neuronal cells derived from different areas of the nervous system. It is worth mentioning that the intracellular pathways activated by NGF binding to TrkA in non-neuronal cells are similar to those described in neuronal models, although they regulate other cell functions [44]. For example, in masts cells, NGF acts as a chemotactic factor by binding to TrkA and activating MAPK and PI3K signaling [45]. However, in monocytes, NGF binding to TrkA plays a key anti-inflammatory role by activating the PI3K pathway and inhibiting the synthesis of inflammatory cytokines [46].

Loss-of-function mutations in NTRK genes can result in class IV hereditary sensory and autonomic neuronal disorders such as Congenital Insensitivity to Pain with Anhidrosis (CIPA) a rare autosomal recessive disorder characterized by insensitivity to noxious stimuli and variable intellectual disability (ID) [47]. TrkA receptors are involved in pain sensation and TrkA misfolding and aggregation induced by mutations results in impaired ability to feel pain or sense differences in temperature [48, 49].

1.3.2 p75 Neurotrophin Receptor

The expression of TrkA is considered necessary and sufficient to elicit NGF biological responses, but the role and contribution of p75 neurotrophin receptor (p75NTR) to NGF signaling and function is still debated and far from being univocally defined. For a long time, the role of p75NTR was considered limited to facilitating TrkA functions and enhancing its specificity.

p75NTR belongs to the tumor necrosis factor receptor (TNFR) superfamily that includes FAS, CD40, CD27 and RANK, all involved in regulating immune responses, inflammation, cell survival and whose dysregulation is implicated in various pathological conditions, including cancer, autoimmune and allergic diseases [50].

p75NTR is encoded by the NGFR/p75NTR gene, located on the long arm of chromosome 17 (17q21.33). At least two isoforms of NGFR/p75NTR exist: a full-length isoform and a shorts isoform (s-NGFR/p75NTR) both of which are expressed in nerve cells **(Figure 3A)** [51]. The full-length isoform includes six exons and five introns [52]. The resulting protein is a single transmembrane-spanning protein with an amino-terminal extracellular domain (ECD) that contains four cysteine-rich motifs (CDR1-CRD4), a transmembrane domain, and a carboxy-terminal intracellular domain (ICD) formed by a juxtamembrane domain (Chopper) and a globular death domain (DDp75NTR) [53] **(Figure 3B)**. The s-NGFR/p75NTR isoform results from alternative splicing of exon III of the NGFR/p75NTR locus which encodes for the CDR 2–4 and consequently lacks the neurotrophin-binding site. For this reason, while the full-length isoform can bind all known neurotrophins, the s-NGFR/p75NTR isoform does not interact with several molecules including Nogo, sortilin and Trk [54].

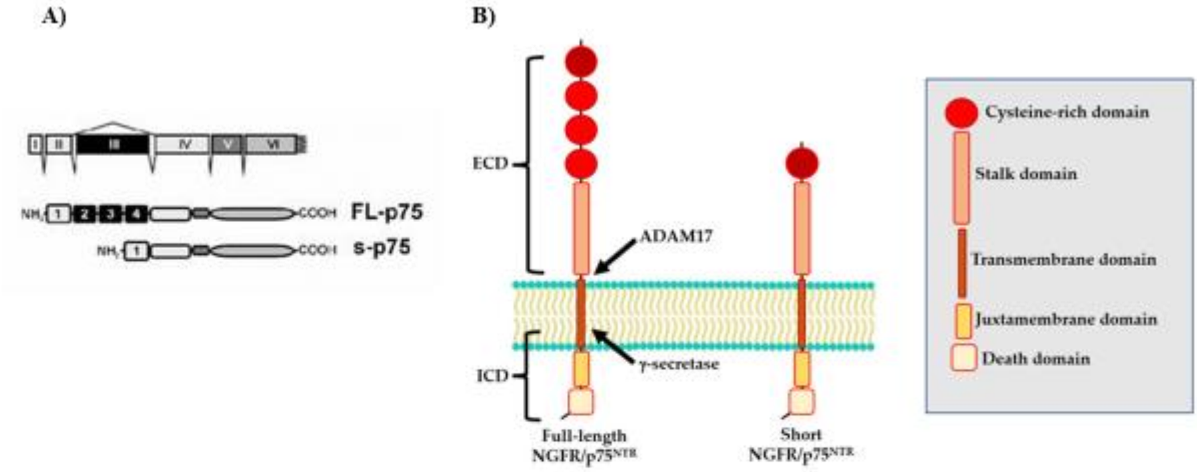

Figure 3. Structure of NGFR/p75NTR isoforms. A) The s-p75NTR transcript is generated by alternative splicing of exon III of the p75NTR locus. The protein encoded by s-p75NTR lacks three of the four Ig-like domains comprising the NGF-binding site of FL-p75NTR. B) P75NTR prtotein structure domain [54]

p75NTR belongs to TNF receptor superfamily (TNFRSF) but it is an atypical TNFRSF protein. The receptors of the TNFRSF differ for the number of cysteine-rich domains (CRD) in the extracellular domain (ECD) and the presence or not of a special domain in the intracellular region, called death domain (DD) [55]. The receptors without DD are called survival receptors and include TNFR2, CD40, CD30, OX40, LTβR, RANK, and TACI. p75NTR like TNF-R1 and Fas-R contain an extracellular domain with four cysteine-rich domains (CRD) and a death domain (DD) in the intracellular region. Receptors of the TNFR superfamily do not contain an enzymatic activity. Instead, activation takes place by recruitment of signaling proteins to the intracellular regions [56] [57]. p75NTR, like TNF-R1 and Fas-R, contains an extracellular domain with four cysteine-rich domains (CRD) and a death domain (DD) in the intracellular region. While TNFRSF proteins are activated by trimeric TNFSF ligands, p75NTR forms dimers activated by dimeric neurotrophins that are structurally unrelated to TNFSF proteins. In addition, although p75NTR shares with other members the interaction with the TNF receptor-associated factors to activate the NF-κB and cell death pathways, p75NTR does not interact with the DD-containing proteins FADD, TRADD, or MyD88. By contrast, the DD of p75NTR is able to recruit several protein interactors via a full catalog of DD interactions not described before in the TNFRSF. p75-DD forms homotypic symmetrical DD–DD complexes with itself and with the related p45-DD; forms heterotypic DD–CARD interactions with the RIP2–CARD domain, and forms a new interaction between a DD and RhoGDI [57].

Thus, p75NTR does not harbour intrinsic enzymatic activity and its signalling depends on the recruitment of intracellular interactors. Before receptor activation by neurotrophins or other signals, the p75NTR-DDs could form a stable dimer. Conformational changes in the p75NTR-ECD propagate to the p75NTR-DD through the disulfide-bonded p75 NTR-TMD and destabilize the p75NTR-DD homodimer, leading to separation of the p75NTR-DD dimer and opening of active sites on the p75NTR-DD surface for recruitment of intracellular interactors for diverse signalling pathways [58]

All these features, in addition to its promiscuous interactions with several ligands and coreceptors, the dimeric nature of its transmembrane domain and its “special” juxtamembrane region, make p75NTR a truly stranger in the TNFR superfamily Moreover, the death domain of Fas and TNFR self-assembles, while that of p75NTR does not [59]. **(Figure 4)**

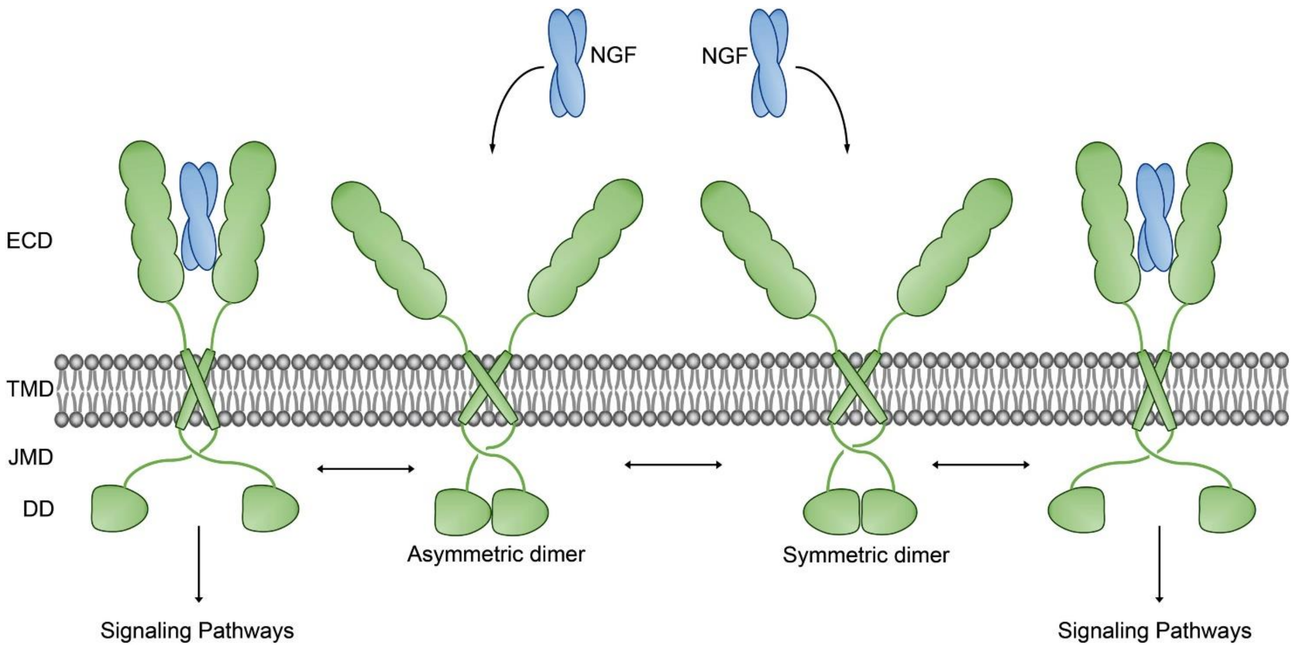

Figure 4. Conformational features of p75NTR [57]

Another key characteristic of p75NTR is its regulated proteolysis, which is an important mechanism for modulating its signaling functions [60]. The first step in the sequential cleavage of p75NTR is mediated by α-secretase, primarily ADAM17 (A Disintegrin and Metalloproteinase 17), also known as TACE (TNF-α converting enzyme). The cleavage occurs in the extramembrane region, close to the transmembrane domain, and it releases the extracellular domain (ECD) into the extracellular space. The remaining fragment, termed the C-terminal fragment (CTF), consists of the transmembrane domain and intracellular domain (ICD). Subsequently to the α-secretase cleavage, the remaining C-terminal fragment of p75NTR is cleaved by γ-secretase, a multi-subunit protease complex, within its transmembrane domain. This cleavage releases the intracellular domain (ICD) into the cytoplasm, which can then translocate to the nucleus interacting with signaling molecules such as RhoA, a GTPase involved in regulating the cytoskeleton, which influences neurite outgrowth and axon regeneration. In some contexts, the p75NTR ICD can also activate apoptotic pathways. For example, it interacts with adaptor proteins such as NRAGE or NADE, which promote apoptosis in neurons under certain conditions and many other funciotns that needed to be investigated. On the other hand, p75NTR ectodomain (ECD) shedding have several functions such to generate soluble decoy receptors that scavenge proNGF, the specific p75NTR ligand, in the extracellular space but, given the right setting, it might also facilitate rather than impede p75NTR-mediated death signals. In some cases, the soluble ECD may act as a signaling molecule itself, though this function is less well understood. In the last decades, it was highlighted the role of p75NTR-ECD as a biomarker in some pathologies considering that it is possible measure ECD excretion in different biological fluids, such as blood, cerebrospinal fluids (CSF) and urine, revealing that there is a great ECD accumulation in neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis, age-related dementia, schizophrenia, and diabetic neuropathy [61].

p75NTR knockout mouse was originally created by deleting **exon III** [62]; thus the short form of p75NTR (s-p75NTR) could still be detected in these mice. The exon 3 knockout mice express a p75 protein variant (s-p75) consisting of one cysteine-rich domain, stalk domain, transmembrane domain and intracellular domain. Primary Schwann cells obtained from p75NTR exon 3 knockout mice express the s-p75 variant that cannot bind neurotrophins but interacts with Trk receptors [51]. p75NTR exon 3 KO mice are viable but smaller than wild-type littermates. Increased skin infections, loss of nails and hair follicles at distal extremities have been observed as major phenotype. KO mice lack 50% of sensory neurons that require more NGF for survival due to 2-3 fold decreased sensitivity to NGF. Loss of sensory neurons mainly resulted in a decreased innervation of the skin. Similarly, impaired heat sensitivity is consistent with decreased cutaneous innervation. Further defects include lack of 40% of dorsal root ganglia (DRG) neurons and Schwann cells; a 40% smaller cross-section of the sciatic nerve.

An alternative mutant mouse was created by deleting **exon IV**, therefore it lacks the transmembrane domain [51]. In 2004, it has been reported that a functional fragment of p75NTR is still produced in exon 4 knockout mice. When overexpressed, this fragment associates with the membrane and it is able to trigger an apoptotic signal [63]. In general, exon 4 KO mice show a more drastic phenotype compared to exon 3 KO mice. They are much smaller than WT mice especially during postnatal period. About 40% do not survive beyond the prenatal period. Most of blood vessels have thin walls and leak blood. The exon 4 KO mice lack 54% of DRG neurons and 61% of the Schwann cells. The cross-section of the sciatic nerve is significantly (54%) smaller compared to WT. However, understanding the phenotype of the exon IV mutants is complicated by the fact that the targeting strategy created a cryptic truncated protein encoding an extracellular stalk with the entire transmembrane and intracellular domains of the receptor [63]. Since expression of the intracellular domain of the receptor can initiate signaling independent of ligand [64], some phenotypic characteristics of this mouse may be due to the expression of this fragment that can activate p75NTR intracellular signalling. Clearly, results from using either of these genetically altered mice need to be interpreted with caution, and further studies are needed to understand the role of s-p75NTR. **(Figure 5)**

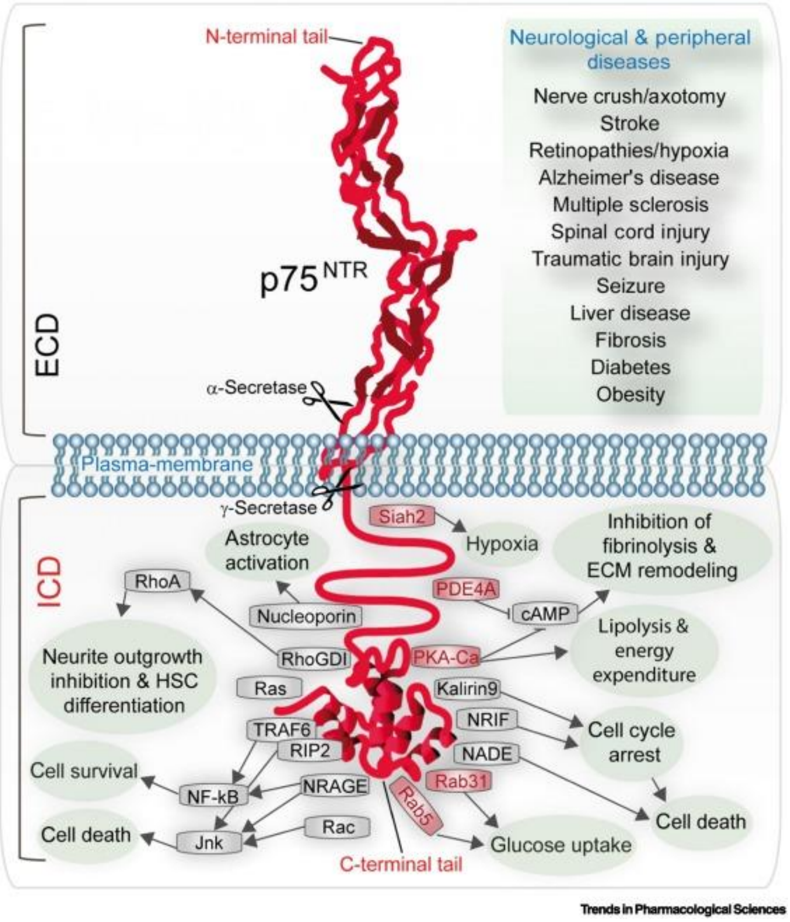

Figure 5. Protein interaction of the p75 neurotrophin receptor. [65]

1.3.3 Cytosolic Factors in p75NTR intracellular pathways

As previously described, p75NTR is a multifaceted receptor involved in neuron survival, apoptosis, and axonal growth. The receptor's function is highly context-dependent, and it can trigger opposing cellular responses, from promoting cell survival to inducing apoptosis, based on its interactions with neurotrophins, coreceptors and cellular conditions. One group of p75NTR interactors is the family of TNF receptor-associated factors (TRAFs). TRAF family proteins are distinguished by a conserved C-terminal domain that is responsible for their oligomerization and interactions with the cytoplasmic domains of TNF receptor family members [66]. TRAF1–6 have been reported to associate with p75NTR, with TRAF2, 4, and 6 shown to modulate p75NTR-induced cell death via interactions with the ICD of the receptor [66] [67]. However, the role of TRAF6 in p75NTR signaling has been the most thoroughly studied. TRAF6 associates with p75NTR in a ligand-dependent manner [68] and mediates signaling from the receptor to both JNK and NF-κB [69]. Sympathetic neurons from *traf6 -/-* mice fail to activate JNK in response to BDNF binding to p75NTR and fail to undergo apoptosis [69]. Furthermore, there is reduced developmental cell death in the superior cervical ganglia in mice *traf6 -/-* relative to the wild type, indicating that TRAF6 is essential for p75NTR-mediated apoptotic signaling *in vivo*. Another intracellular binding partner of p75NTR that is linked to JNK activation is the neurotrophin receptor-interacting MAGE homolog, NRAGE (also known as Maged1 and dlxin [70]. NRAGE contains a melanoma-associated antigen (MAGE) domain, which is a region of homology defining the MAGE family of proteins. The function of the MAGE proteins is poorly understood, but many have been implicated in the regulation of cell cycle and apoptosis. Ectopic expression of NRAGE along with p75NTR in a sympathetic precursor cell line enabled NGF-dependent cell death, thereby implicating this interactor in the apoptotic pathway activated by p75NTR [30]. Overexpression of NRAGE in PC12 cells led to potent activation of JNK, release of cytochrome c from mitochondria, and the induction of caspases -3, -6, and -9, ultimately resulting in cell death [70]. These results suggested that NRAGE could be involved in p75NTR-mediated stimulation of JNK. Corroborating evidence came from analysis of *nrage-/-* mice: p75NTR-induced JNK activation in *nrage-/-* sympathetic neurons was significantly reduced compared to wild-type neurons [71]. Furthermore, the null animals have an increased umber of neurons in their superior cervical ganglia, like *p75NTR-/-* mice, and sympathetic neurons isolated from *nrage-/-* mice were resistant to p75NTR-mediated apoptosis [71]. These results suggest a function for NRAGE as an adaptor protein, linking the receptor to JNK activation and apoptosis. Whether NRAGE, TRAF6, and NRIF form a complex or function independently to regulate the kinase remains an open question; however, they may function at different stages of the cascade to affect the kinetics of JNK activity. It should be noted that sequestering the anti-apoptotic factor XIAP [72] and promoting degradation of the anti-apoptotic transcription factor Che1 [73] have also been suggested as mechanisms through which NRAGE affects cell survival, though these interactions have not been studied in the context of p75NTR signaling. Another mechanism through which p75NTR has been suggested to regulate JNK involves production of the lipid signaling molecule ceramide **(Figure 6)**. When the field was searching for evidence of signaling by p75NTR, a NGF-mediated increase in ceramide levels through activation of neutral sphingomyelinase in T9 glioma cells was one of the first signals detected [74].

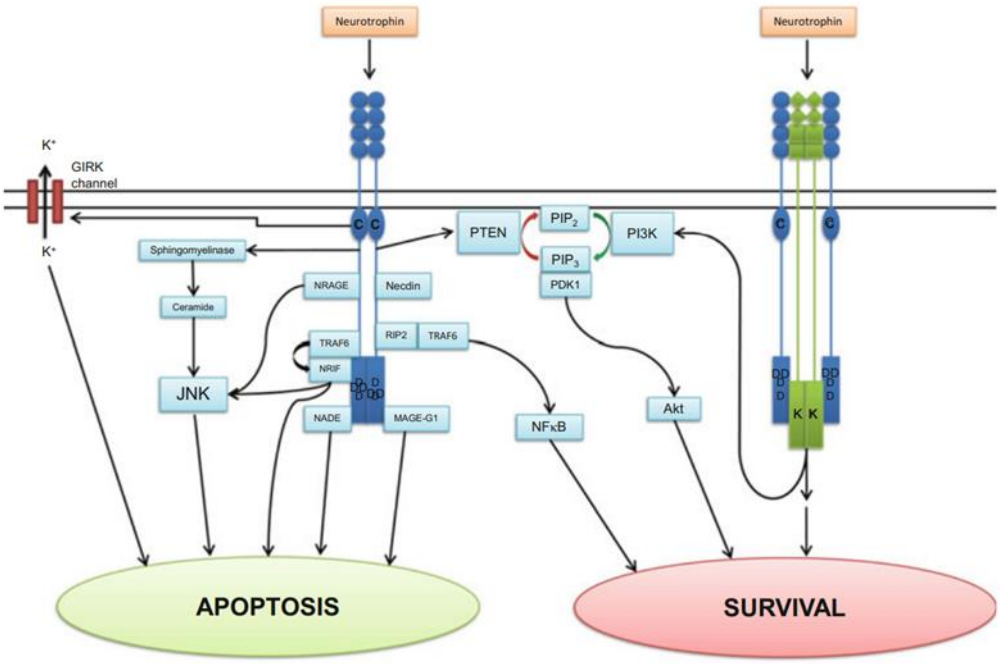

Figure 6. Signaling pathways mediated by p75NTR that regulate cell survival and apoptosis. In response to neurotrophin binding, p75NTR promotes JNK activation via interactions with NRAGE, TRAF6, and NRIF, thus leading to apoptosis. Activation of JNK by p75NTR also occurs through induction of sphingomyelinases. The chopper domain of p75NTR promotes apoptosis by facilitating depletion of internal K+ through GIRK channels. Other cytosolic interactors contribute to p75NTR-mediated cell death, including NADE, MAGE-G1, and Necdin. In response to pro-neurotrophins, p75NTR inhibits Trk-mediated survival signaling via induction of PTEN and the resultant inhibition of PI3K-Akt survival signaling. Promotion of cell survival by p75NTR is facilitated by its interactions with Trk receptors which enhance Trk-mediated PI3K-Akt survival signaling, as well as other Trk-mediated survival pathways. P75NTR may also promote survival via activation of NFκB, possibly through associations between RIP2 and TRAF6 (abbreviations: DD p75NTR death domain, C p75NTR chopper domain, K Trk receptor tyrosine kinase domain) [72]

1.3.4 proNGF binding to p75NTR

The initial discovery that p75NTR can induce programmed cell death was somewhat puzzling, as in vitro studies indicated that relatively high concentrations of neurotrophins were needed to induce apoptosis, and in certain cell types, cross-reactivity of neurotrophins with Trk receptors could potentially promote a pro-survival signal. The discovery that proneurotrophins do not activate Trk receptors [66] led to the finding that proNGF binding to p75NTR induces significant cell death at sub-nanomolar concentrations [17]. The binding affinity (Kd) of proNGF to p75NTR has been reported to be 18.3 nM for human p75NTR and mouse proNGF [75]. Thus, proteolytic processing determines the functional fate of nascent neurotrophins, with immature precursors selectively triggering p75NTR-mediated cell death and mature forms activating either p75NTR or Trk receptors, depending upon the cellular context. Proneurotrophins induce programmed cell death by binding to a high affinity receptor complex constituted by p75NTR and its co-receptor Sortilin, a member of the Vps10p-domain receptor family [76]. Mammalian members of the Vps10p family, that includes Sortilin, SorLA, and SorCS-1, -2, and -3, are type I transmembrane receptors with multifunctional roles that involve the modulation of protein sorting and trafficking, as well as the regulation of signal transduction[77]. Proneurotrophins bind to Sortilin via their pro-domain and to p75NTR by their mature domain, thus facilitating the association of these two receptors to initiate programmed cell death. Following initial reports that Sortilin mediates neurotrophin-induced cell death, *in vitro* studies have indicated that Sortilin is required for developmental p75NTR-mediated cell death *in vivo*. [76]. For example, mice lacking Sortilin have a reduction in the developmental apoptosis of retinal ganglion cells that is indistinguishable from that of p75NTR-deficient mice [78]. However, Sortilin may not be required for all p75NTR-mediated cell death, as these mice did not have defects in the apoptosis of sympathetic neurons during the developmental time period in which p75NTR-mediated death is known to occur [78]. Loss of Sortilin did, however, impair age-related degeneration of these neurons, suggesting that proneurotrophins may not have been involved in the early development of the sympathetic neurons, but do have a role in their loss during aging. [66]

1.3.5 p75NTR activation influences key processes in nervous system

In addition to its critical role during neurodevelopment, p75NTR is a stress-activated receptor that stimulates the death of cells within injured tissue. Though the receptor is downregulated in most regions of the nervous system after early postnatal development, re-expression of p75NTR occurs in response to many forms of cellular damage. For example, increases in p75NTR expression have been reported following neuronal axotomy [79], mechanical damage [80], elevated intraocular pressure,[81] seizures [82], and focal ischemia [83]. Beyond measuring increases in expression of the receptor, multiple studies have demonstrated that activation of p75NTR signaling in the degenerating neurons correlates with their apoptosis that is abolished by function-blocking p75NTR antibodies[84]. Although a number of studies in CNS and PNS indicated that p75NTR promotes neuronal death after injury, the role of proneurotrophins was not known. Only recently it has been demonstrated that proNGF is produced and secreted *in vivo* after CNS and PNS injury inducing p75NTR-dependent apoptosis of spinal cord oligodendrocytes[85]. Disruption of this interaction by infusion of neutralizing antibodies for proNGF prevents the injury-induced apoptosis. These experiments provided the first evidence that proNGF is a pathophysiological ligand that induces apoptosis together with p75NTR in response to neuronal damage. In addition to regulating cell death following injury, p75NTR signaling has been suggested to contribute to neurodegeneration caused by a number of diseases. Among these disorders, the link between p75NTR and Alzheimer's disease (AD) has been most studied. p75NTR is expressed at high levels in cholinergic neurons of the adult basal forebrain, a population of neurons that undergoes severe degeneration early in the progression of AD pathology. *In vitro* studies have indicated that amyloid beta 1–42 (Aβ), the main component of plaques commonly found within brains of AD patients, is a pro-apoptotic ligand for p75NTR [86]. These findings have led to the hypothesis that activation of p75NTR by Aβ contributes to neurodegeneration caused by AD. Moreover, p75NTR may contribute to degeneration of motor neurons during the progression of amyotrophic lateral sclerosis (ALS). Though p75NTR is downregulated in motor neurons of the spinal cord during the perinatal period, re-expression of the receptor was detected in spinal motoneurons of an ALS mouse model [87]as well as in spinal cord samples from human patients with ALS [88]. Degeneration of dopaminergic neurons in Parkinson's disease (PD) involves p75NTR that induces selective apoptosis of dopaminergic neurons since the activation of p75NTR signaling inhibits the expression of transcription factors which are essential for the survival of dopaminergic neurons thus contributing to thenprogression of PD. In addition to these neurodegenerative conditions, p75NTR has been shown to be involved in the pathology of other neurological diseases: spongiform encephalomyelopathy [89],diabetes-related impairment of neovascularization [90], and psoriasis [91], among others. The abundance of experimental evidences linking p75NTR to such a variety of diseases indicates that this receptor may function as a stress-induced apoptotic signal that is activated by a mechanism common to all of these pathological conditions.

1.3.6. Promotion of Cell Survival

Despite its currently known role in eliciting programmed cell death, early studies of p75NTR demonstrated that in a variety of cellular contexts the receptor has the opposite function: to promote cell survival. Though p75NTR-induced apoptosis has been more widely studied, the receptor has been demonstrated to promote cell survival in a wide variety of cell types. *In vivo* evidence, using p75NTR-/- mice, revealed significant loss of sensory innervation of limbs. Subsequently, the number of neurons in the dorsal root ganglia (DRG) was reported to be reduced by 50–75 % in the knockout mice. Numerous reports have suggested that p75NTR promotes survival in a wide range of cell types, with the cooperation with the Trk family, leading to a high affinity receptor complex or enhancing Trk signaling itself by activation of the pro-survival kinase Akt. Treatment of PC12 cells with NGF in the presence of an antibody that blocked binding to p75NTR inhibited the activation of Akt [92]. Otherwise, cleavage of p75NTR was induced by Trk activation promoting phosphorylation of TACE, which activated the protease and lead to cleavage of p75NTR, which was necessary for potentiation of neurotrophin-induced survival signaling [93] **(Figure 6)**.

Apart from enhancing Trk survival signaling, there is evidence that p75NTR can activate an independent pro-survival signal; for example, selective activation of p75NTR prevented the death of certain neuroblastoma [94] and breast cancer cells [95], of hippocampal neurons treated with NMDA [92], and of both sensory neurons [96] and cortical subplate neurons deprived of trophic support. In addition, the receptor appears to play a protective role after certain injuries; e.g., p75NTR/ mice have increased death of primary auditory neurons following acoustic trauma[97]. The pro-survival effects of p75NTR appear to be more of a modulatory signal, as they are not as [98]potent as the effects of tyrosine kinases like the Trks. The molecular mechanisms by which p75NTR promotes survival independent of the Trk receptors are not fully understood; however, one downstream pathway that has been identified involves the transcription factor nuclear factor kappa B (NFκB). NFκB is best characterized for its role in the immune system, where it is activated by many cytokine and Toll-like receptors, leading to upregulation of other cytokines and pro-survival genes. The activation of NFκB by p75NTR was first reported in Schwann cells [99] and has since been demonstrated in a variety of cell types, including Schwannoma cells primary Schwann cells [100], trigeminal neurons [101], and hippocampal neurons [102]. NFκB exists as a dimer, held in the cytosol through binding to its inhibitor IκB. The transcription factor is activated through phosphorylation of IκB by the IκB kinase (IKK) complex, leading to proteasomal degradation of the inhibitor and release of the NFκB dimer to translocate into the nucleus. As mentioned above, neurotrophin binding to p75NTR can recruit members of the TRAF family, which activate the IKK complex; specifically, TRAF6 was shown to mediate activation of NFκB, as Schwann cells from traf6-/- mice did not respond to p75NTR activation [69]. Another well-established activator of NFκB is Akt. Although evidence suggests that p75NTR enhances Trk activation of Akt, as discussed above, p75NTR has also been reported to promote Akt activation in a manner that was independent of Trk signaling in hippocampal neurons[103], melanoma cells, and mutant PC12 cells lacking TrkA [104]. Therefore, NFκB may be among the downstream pro-survival signals activated by p75NTR in some contexts.

1.3.7 p75NTR as a pharmacological target in neurological diseases

Based on the evolving knowledge of the multiple roles of p75NTR, the expression of this receptor and the tissutal presence of its ECD or soluble p75NTR could be considered as biomarkers to evaluate the severity of diseases and as possible target for the treatment of numerous neurological disorders [105] . Several prototype inhibitors of p75NTR have been tested to assess their ability to promote neuronal survival and functional recovery. These compounds represent possible pharmacological tools for attenuating neurodegeneration and cell death as they abolish activation-induced neurotoxicity of neurotrophins via modulation of p75NTR signalling. These inhibitors act through various modes of action and at present four major classes of p75NTR-modulating agents are considered for pharmacologic manipulation: 1) modulators of p75NTR expression, 2) inhibitors of p75ICD interaction with its partners, 3) agents blocking p75NTR cleavage, and 4) agents blocking ligand binding to p75ECD. More comprehensive and in-depth investigations on p75NTR-based drug development are required to shed light on their efficacy **(Figure 7).** More specifically, the LM11A-31 represents the best prospect for a future drug to be used in clinic approach to inhibit the biological action of p75NTR. LM11A-31 is a small molecule designed as a therapeutic agent targeting p75NTR, derived from the loop 1 domain of NGF. Its chemical formula is $C_{12}H_{27}Cl_2N_3O_2$ or (2S,3S)-2-Amino-3-methyl-N-[2-(4-morpholinyl)ethylpentanamide dihydrochloride with a molecular weight of 316.26. LM11A-31 is a structural mimetic compound designed to interact with the extracellular domain of p75NTR, specifically inhibiting the interaction between p75NTR and its ligands, including proNGF and proBDNF without interfering with Trk receptor activity. It selectively inhibits pro-apoptotic signaling cascades associated with p75NTR without affecting neurotrophin-Trk receptor-mediated signaling, ensuring neuroprotective effects while minimizing side effects. More specifically, LM11A-31 prevents the activation of downstream signaling pathways that lead to apoptosis, neuroinflammation and axonal degeneration [65]. LM11A-31 is optimized for oral administration, providing ease of use in chronic conditions, and for effectively crossing the blood-brain barrier, to achieve therapeutic concentrations in the central nervous system (CNS) [106]. LM11A-31 has a sufficient half-life to enable daily dosing schedules, though specific pharmacokinetic parameters vary between studies and animal models. Moreover, LM11A-31 is predominantly metabolized in the liver and although its metabolic pathways are not extensively described in literature they result in non-toxic metabolites. LM11A-31 has been extensively studied *in vitro* and *in vivo* in various disease models, demonstrating promising neuroprotective potential. LM11A-31 crosses the blood-brain barrier based on rodent studies [106].

In *in vitro* experiments it has been demonstrated that LM11A-31 reduces proNGF-mediated apoptotic signaling in cultured neurons, attenuating markers of oxidative stress and mitochondrial dysfunction. In transgenic mouse models of Alzheimer's (e.g., 3xTg-AD mice), LM11A-31 improves synaptic density and cognitive function, reduces amyloid-beta accumulation and tau hyperphosphorylatio and mitigates neuroinflammation by decreasing activation of microglia and astrocytes [107, 108]. Moreover, in other mouse model of Alzheimer's disease (APPL/S mice), LM11A-31 treatment (0, 10, or 50 mg/kg/day, orally) for 3 months prevented deficits in novel object recognition and Y-maze performance [106]. In these mice, neuritic dystrophy was present in the basal forebrain, hippocampus, and cortex, but was significantly reduced by LM11A-31 treatment, with no effects on amyloid levels. In a mouse model of Huntington's disease (R6/2 mice), treatment with LM11A-31 (50 mg/kg/day, oral gavage, 5-6 days/week) for 7-8 weeks starting at 4 weeks of age before cognitive/motor symptom onset alleviated volume reductions in multiple brain regions, including striatum, globus pallidus, cortex, corpus callosum and contiguous external capsule [107] . However, no treatment effects were seen in the volumes of the hippocampus or thalamus. LM11A-31 treatment diminished increases in plasma cytokine levels, including TNF-α, IL-1β, and IL-6. Moreover, while R6/2 mice treated with vehicle had increased urinary levels of the p75NTR extracellular domain (ECD), a cleavage product released with pro-apoptotic ligand binding, while LM11A-31 treatment reduced this increase. The levels of urinary p75NTR-ECD may represent a novel marker of disease state and therapeutic efficacy in Huntington's disease. LM11A-31 has been tested in Spinal Cord Injury (SCI) both *in vitro* showing that survival and growth of axons in neuronal cultures subjected to mechanical injury or in in vivo demonstrating that in rodent models of SCI, LM11A-31 reduces secondary injury processes such as apoptosis and inflammation. In Amyotrophic Lateral Sclerosis (ALS), LM11A-31 slows disease progression by preserving motor neuron integrity in SOD1-G93A mouse models, improving motor function and extends survival. In a rat model of traumatic brain injury, LM11A-31 treatment (50 or 75 mg/kg/day, i.p.) started at 24 hours after injury (controlled cortical impact) and continued for 21 days significantly improved learning and memory outcomes [109]. In a mouse model of repetitive mild traumatic brain injury, treatment with LM11A-31 (50 mg/kg, i.p.) one hour before the first hit and on days 0, 1, 3, and 7 resulted in better recovery of axonal integrity. LM11A-31 reduces demyelination and axonal damage in experimental autoimmune encephalomyelitis (EAE) models. Also in Peripheral Neuropathy LM11A-31 shows neuroprotective effects in models of diabetic neuropathy and chemotherapy-induced peripheral neuropathy. LM11A-31's selective inhibition of p75NTR prevents neurodegenerative processes without disrupting beneficial neurotrophic signaling. Indeed, its action is preserved across multiple models, reducing apoptosis, inflammation, and neurodegeneration. Moreover, the oral administration facilitates long-term use in chronic diseases. At present, one of the limitation of its use is that the efficacy and safety profiles required careful validation in humans, because as of now there are limited clinical data, most of them deriving from preclinical models. Ongoing clinical trials will provide deeper insights into its therapeutic potential and safety profile in humans. Actually, LM11A-31 is under clinical development by PharmatrophiX. In a randomized double-blind phase 2a clinical trial in in 242 subjects mild-to-moderate Alzheimer's patients, LM11A-31 has been demonstrated to have a plasma half-life of 1 hour (brain half-life of 3-4 hours in mice). Doses of 200 mg/day and 400 mg/day of LM11A-31 have been tested [110]. In rodent studies, doses ranging from 5 to 100 mg/kg have been used, with 50 mg/kg being the most common [111] [108] . Most studies used the oral gavage route. For *in vitro* testing, LM11A-31 was dissolved in water prior to dilution in culture medium. For *in vivo* testing, LM11A-31 was dissolved in water at a concentration of 5 mg/ml and stored at −20 °C.

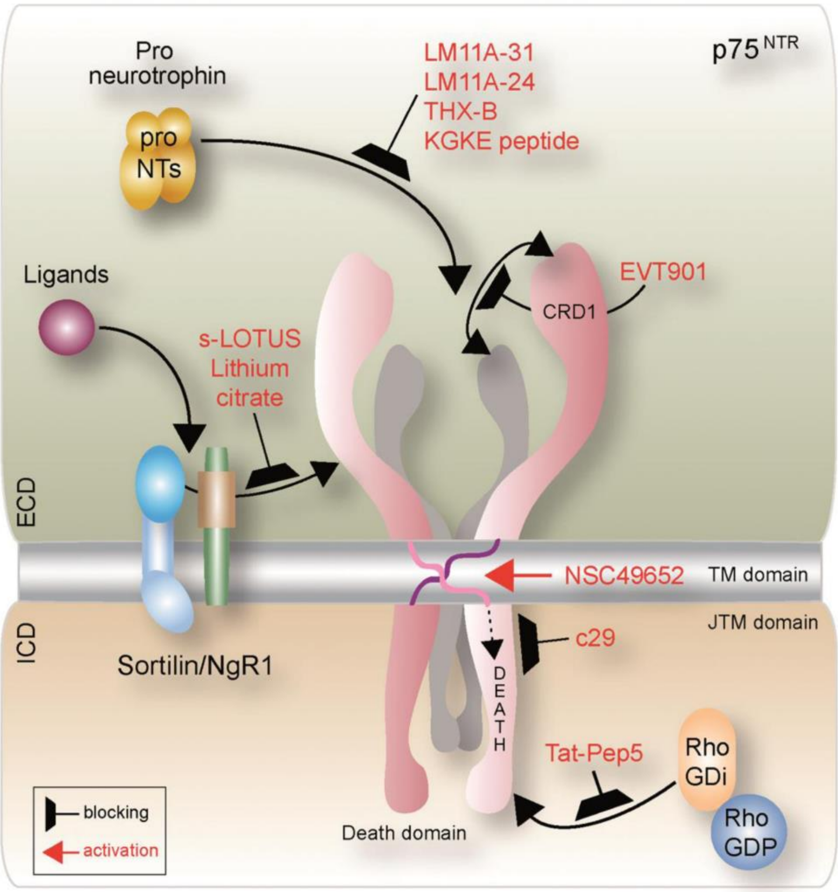

Figure 7. Mechanism of action of p75NTR - modulating small molecule compounds. LM11A-31, LM11A-24, THX-B and the KGKE peptide or homologous sequence inhibit proNT binding to p75NTR, EVT901 interacts with the p75NTR CRD1 and inhibits p75NTR pre-oligomerization, NSC49652 interacts with the p75NTR TM domain and induces conformational changes and p75NTR activity, lithium citrate prevents the association of the p75NTR-sortilin receptor complex, s-LOTUS inhibits the interaction between NgR1 and p75NTR, the c29 peptide inhibits p75NTR cytoplasmic juxtamembrane death signaling, and the TAT-Pep5 peptide inhibits p75ICD and Rho-GDI. Abbreviations: p75NTR; p75 neurotrophin receptor, ECD; extracellular domain, TM domain; transmembrane domain, ICD; intracellular domain, JTM domain; juxtamembrane domain, CRD1; cysteine rich domain 1, NgR1; Nogo receptor 1. [105]

2 NGF and the immune system

Although for some time after its discovery the action of NGF was considered restricted only to the nervous system, already in her Nobel lecture Prof. Levi-Montalcini hypothesized a broader physiological action of NGF beyond neurobiology (RLM, Nobel lecture, December 8, 1986) predicting a significant role of NGF in the immune system regulation. Indeed, subsequent studies demonstrated that NGF receptors are expressed in immune organs and in a variety of immune cell populations, allowing NGF to modulate cell differentiation and regulate immune response. As a growing number of studies have shown, an enhanced production of NGF characterizes inflamed tissues of patients with inflammatory and autoimmune diseases and inflammatory mediators inducing NGF synthesis in a variety of cell types. Although the dynamic regulation of NGF synthesis seems to be a common feature of chronic inflammatory diseases, the reasons why NGF concentrations are enhanced and how this can affect inflammatory responses, and the course of the diseases need to be better investigated

2.1 NGF and NGF receptors sistem influence development and differentiation of the immune system

During embryo development, the expression of NGF receptors is finely modulated in primary and secondary lymphoid organs [112] and declines during postnatal life [113]. In bone marrow and in the thymus, NGF receptor expression has been found in stromal cells, which release specific growth factors and signals [113] regulating the correct differentiation of myeloid and lymphoid precursors. In the thymus, the expression of NGF and of both its receptors is very high in the final stages of embryo development and in the early postnatal period and then decreases with age [112, 114]. Similarly to what has been described in bone marrow, there is also a gradient of expression of NGF receptors during embryo development in the bursa of Fabricius [113], the specific avian organ where the maturation and differentiation of B-cells occurs. During the post-hatching period, TrkA is expressed in epithelial cells of the follicle, in the interfollicular epithelium of the bursa of Fabricius and in blood vessels [113]. The local production of NGF seems to be involved in follicle differentiation *in vivo*, since NGF administration accelerates follicle formation in the chick embryo and influences the survival of bursal cells.

The expression of NGF receptors also characterizes secondary lymphoid organs (spleen, lymph nodes and mucosa-associated lymphoid tissues) where antigen-presenting cells activate lymphocyte responses and effector cell differentiation. The spleen is composed of two different tissues: the white pulp, responsible for the production and growth of immune and blood cells, and the red pulp, which filters microorganisms and cellular debris from the blood and removes older erythrocytes from circulation [115]. The expression of p75NTR and TrkA is localized primarily in the stroma of the spleen, with some expression in splenocytes [116] and in spleen mononuclear immunocompetent cells [114]. In rats, during spleen development, the level of p75NTR mRNA increases [116] and is expressed mainly by splenic nerve fibers and in a subpopulation of dendritic cells [117]. In lymph nodes and in mucosa-associated lymphoid tissues, TrkA is present in follicular dendritic cells, in blood vessel walls, in cryptic tonsillar epithelium and in several monocyte-derived cells including epithelioid and multinucleated Langhans' cells and interdigitated reticular cells. The expression of p75NTR has been described in follicular dendritic cells of lymphoid follicles, interdigitated reticular cells, periarteriolar macrophages, endothelial sinusal cells and nerve endings, and is also found in moderate levels in dendritic cells of tonsillar follicles. [118, 119].

The picture emerging from all these studies is that the expression of NGF receptors characterizes both stromal and immune progenitors and is finely regulated during development and post-natal life. Moreover, the constant production of NGF in lymphoid organs [112, 114] seems to be important for regulating the differentiation of hematopoietic stem cells.

CD34 positive hemopoietic stem cells express both p75NTR and TrkA maintening the expression *in vitro* even in the absence of inducing factors. The possibility that NGF may be a key factor for haemopoiesis is supported by the fact that hematopoietic stem cells produce their own NGF in an autocrine fashion, probably regulating anti-apoptotic genes. [120-122]. In CD34+ cells, NGF can act as a paracrine or autocrine factor, influencing cell survival, differentiation, and proliferation. As demonstrated in human cord blood cells, a gradient of TrkA and NGF expression exists that is highest in CD34-positive cells, reduced in cord blood mononuclear cells, minimal in mononuclear cells isolated from adult peripheral blood, and it further declines with age. Thus, NGF expression is at its highest levels in the more undifferentiated cells, declines during lineage differentiation of immune cell and re-increases during cell activation. This modulation of NGF expression suggests that NGF may play different functions depending on the state of differentiation and functional activity of the immune cells.

In vitro studies have shown that the administration of NGF in semisolid cultures increases long-term survival of human hematopoietic cells and promote the commitment toward specific lineages in human and murine myeloid progenitor cells.[123]. TrkA mediates functional responses to NGF in immature hematopoietic cell lines and mouse Bone Marrow (BM) precursor cells activatining a signaling cascade which promotes cell survival and proliferation through MAPK/ERK and PI3K/AKT[120] On the contrary, in CD34+ cells p75NTR expression might modulate apoptosis especially when NGF levels and TrkA expression are both impared. Support for the possible pro-survival role of NGF trough TrkA in hematopoiesis is also provided by studies on leukemia showing a constitutive activation of TrkA in blasts that affects survival of the leukemic cells in patients and in animal models.

More recently, a close relationship between p75NTR expression and pluripotency has been described. In fact, p75NTR was shown to be expressed in various types of stem cells and has been used to prospectively isolate stem cells with different degrees of potency. p75NTR is implicated in the regulation of stem cell differentiation in several cell models, including mesenchymal, dental pulp, testis and bone marrow stem cells. This action could be achieved through the induction of a quiescent state, as documented in neuronal and hematopoietic stem cells or in breast cancer cells considering that p75NTR act as a “fate decision” protein that enables stem cells to maintain their potency and its expression is down-regulated as a cell begins its differentiation program [124].

2.2 NGF in Immune System Differentiation and the Immune Response:

2.2.1 NGF and NGF receptor systems in innate Response:

During the activation of innate immune response, *in vitro* studies demonstrated that *granulocytes* and *mast cells* express NGF receptors, produce their own NGF that is released after degranulation, and highlithed the role of these cells in hyperalgesia and neuroimmune interactions. Once released, NGF not only induce maturation and survival [125] but is also a potent degranulation factor for mast cells *in vitro* and *in vivo,* acting as a co-factor with interleukin-3[126].. In activated *basophils*, NGF enhances the synthesis of lipid mediators (leukotrienes) in response to inflammatory stimuli and together with IL-3 and IL-33 acts as a cofactor to induce the release of histamine and enhance the response to IgE. The effect of human NGF on basophil mediator release is similar to that of interleukin-3, interleukin-5, and granulocyte-macrophage colony-stimulating factor [127].. In addition, several studies have shown that NGF is a chemoattractant factor for both granulocytes and masts cells [45]. Besides that, NGF influces the migration of *eosinophils* towards the site of inflammation in the airways regulating the release of peroxidase and IL-4 secretion in these cells [128]. Also eosinophils are able to produce and release their own NGF following inflammatory stimuli activation. In patients with seasonal allergic rhinitis, the concentrations of NGF in nasal secretions correlate with the magnitudes of eosinophilic inflammation in the airway, suggesting a potential clinical implication of NGF in allergic responses [129]. Taken together all these data suggest that the main activity of NGF on cells of the myeloid lineage is to enhance effector functions, such as the release of inflammatory mediators, chemotaxis, and proliferation. **(Figure 9)**

In *monocytes*, in addition to proliferation and survival, NGF influences cytokine release. and protection from apoptosis, by inducing the expression of anti-apoptotic proteins Bcl-2, Bcl-xl and Bfl-1 [130]. Moreover, NGF can promote the differentiation of monocytes into macrophages or dendritic cells, which are critical for tissue repair, phagocytosis of pathogens, and antigen presentation in the immune system. Human monocytes synthesize basal levels of NGF and Calcitonin gene-related peptide (CGRP) that are both up-regulated following lipopolysaccharide (LPS) stimulation. When endogenous NGF is neutralized, the up-regulation of CGRP expression induced by LPS is inhibited. In addition, NGF deprivation in LPS-treated monocytes significantly decreases interleukin 10 (IL-10) synthesis.Endogenous NGF synthesis has a functional role and represent a physiologic mechanism for down-regulating major histocompatibility complex (MHC) class II and CD86 expression and altering the development of immune responses[131]. The possible anti-inflammatory role of NGF has been further studied in monocytes activated with ligands of TLR4 and TLR2. The study demonstrated that TLR activation in human monocytes, upregulates TrkA expression and specifically enhance auto-phosphorylation of TrkA induced by NGF binding that activats intracellular pathways. The NGF-induced phosphorylation of Akt inhibits NF-κB translocation in the nucleus. The inhibitory phosphorylation of GSK3 induced by Akt further prevents NF-κB activation and the NF-κB-dependent transcription of pro-inflammatory cytokine genes. Concomitantly, NGF activation of the PI3K/Akt pathway induces the expression of IL-10 and IL-1 receptor antagonist (IL-1ra), promoting a net anti-inflammatory effect **(Figure 8)**[46].

The binding of NGF to TrkA activates distinct pathways that influence the intracellular signaling induced by inflammatory stimuli with an overall anti-inflammatory action, down-regulating the production of inflammatory cytokines that are pivotal in orchestrating innate and adaptive immune responses. The addition of NGF tilted the balance of the pro- and anti-inflammatory pathways that are simultaneously activated during inflammatory response, potentiating the release of anti-inflammatory mediators such as IL-10. Consequently, changes in TrkA expression levels may affect the physiological anti-inflammatory mechanism mediated by NGF. **(Figure 8)**.

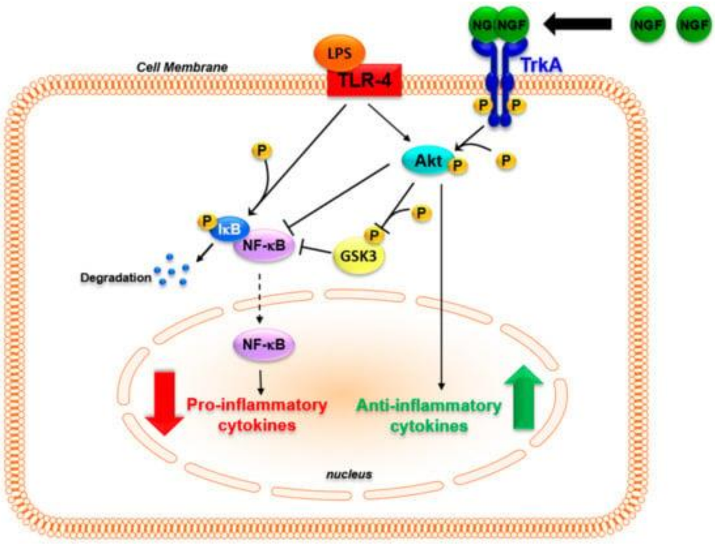

Figure 8. TrkA activation promotes anti-inflammatory pathways. [46]

2.2.2 NGF and NGF receptor systems in Adaptative response:

Studies of mature lymphoid cells have shown that NGF has a proliferative effect on both B and T cells.NGF is produced by CD4+ T-cell clones that express TrkA receptor inducing an upregulation of proliferative response and cytokine expression. NGF and its receptors also have significant functions in B cells: NGF stimulates B-cell proliferation, antibody production, memory B-cell survival, CD40 expression[132]. and causes the differentiation of B cells into immunoglobulin-secreting plasma cells influencing plasma cells survival. NGF stimulates B-lymphocytes to produce IgM, IgA, and IgG and regulates the survival of B-memory cells [44], which rapidly and efficiently counteract pathogens when re-encountering antigens during the secondary response, a key feature of immunological memory. The activation of TrkA pathways in B cells results in apoptosis inhibition through regulation of the expression of anti-apoptotic proteins Bcl-2, Bcl-xl and Bfl-1[123]. **(Figure 9)**

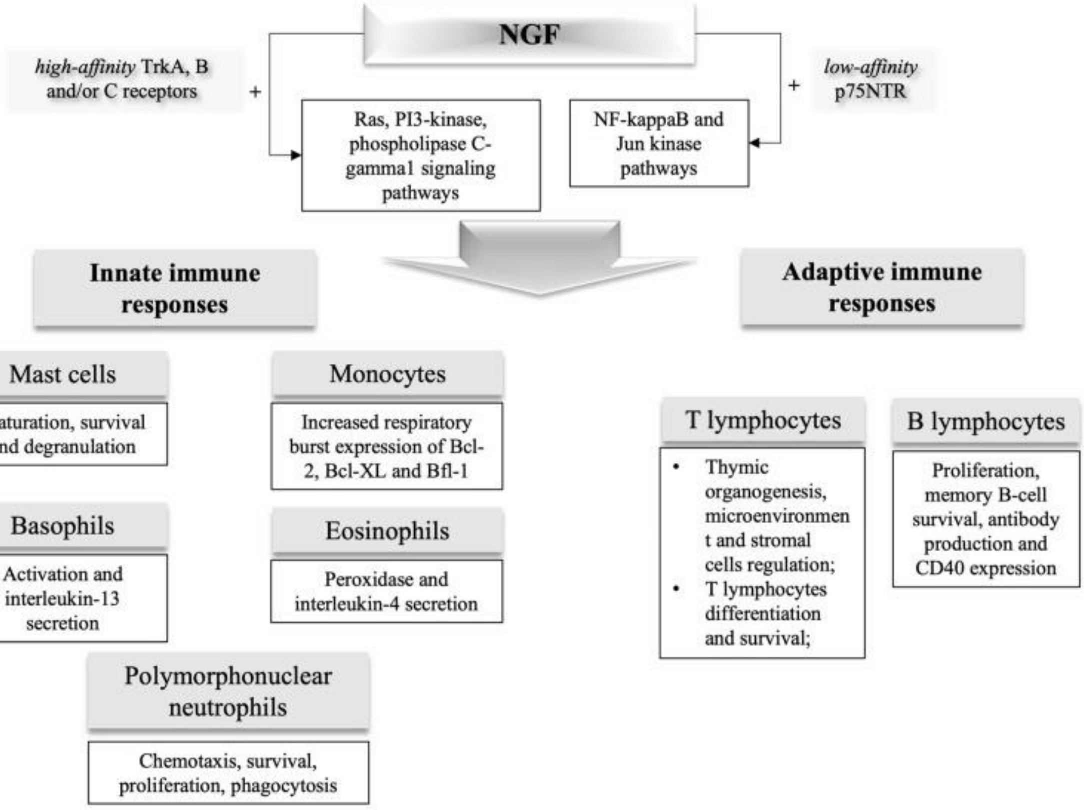

Figure 9. Role of NGF as an immune system modulator. Through different pathways high-affinity Trk A, B, and/or C receptors trigger Ras, PI3-kinase, phospholipase C-gamma1 signaling pathways, while low-affinity p75NTR triggers many paths including those associated with NF-kappaB and Jun kinase. NTs play a major role in many physiological activities including neuroregulation, angiogenesis, immunomodulation, reproduction, and bone tissue regulation. NGF released from tissue mast cells because of nervous, immune, or endocrine stimuli cause innate and adaptive immune responses: mast cells maturation, survival and degranulation; neutrophils chemotaxis, survival, proliferation, phagocytosis; thymic organogenesis, stromal cells, and microenvironment regulation; T-lymphocyte differentiation and survival; B-cell proliferation, memory B-cell survival, antibody production, and CD40 expression; monocytes increased respiratory burst expression of Bcl-2, Bcl-XL, and Bfl-1; eosinophils peroxidase and interleukin-4 secretion; basophil activation and interleukin-13 secretion. NF-kappaB, nuclear factor-kappaB; NGF, nerve growth factor; NTs, neurotrophins; p75NTR, p75 neurotrophin receptor; Trk, tropomyosin-related kinase; PI3, phosphatidyl inositol-3 [132].

3. NGF regulation of inflammation

3.1 In vivo evidence and experimental models.

Studies on inflammatory and autoimmune diseases, which are characterized by an abnormal activation of immune cells and increased production of cytokines, have revealed a localized increase in NGF at the sites of inflammation. Enhanced NGF levels were initially found in the cerebrospinal fluid of multiple sclerosis patients (MS), and it was shown that an increase in NGF closely follows the course of the disease [133] probably exerting a dual role during the MS diseases: NGF promotes both axonal regeneration, survival, protection and differentiation of oligodendrocytes (OGs) and facilitates migration and proliferation of oligodendrocyte precursors (OPs) to the sites of myelin damage [134] but it may also exacerbate inflammation by increasing the activity of immune cells in the CNS.

NGF is elevated in inflamed joints in Reumatoid arthritis patients: the normally low basal production of NGF is enormously up-regulated during inflammatory response [135]. Synovial fluids of patients with RA and circulating serum of juvenile idiopathic arthritis JIA patients [136, 137] are characterized by an increased concentration of NGF. NGFup-regulation in inflamed synovia was demonstrated in studies on different animal models of induced arthritis [138].

The increase of tissutal NGF during synovia inflammation modified the phenotype of innervating neurons, directly regulating the amount and the type of neurotransmitter produced and increasing the expression of transient receptor potential cation channel subfamily V member 1(TRPV1), also known as vanilloid receptor, that mediate pain sensation [139]. As a result, pain sensitivity is increased during inflammation: the properties of the somatosensory neurons change so that the pain threshold decreases, noxious stimuli evoke more acute and prolonged pain (hyperalgesia) and normally innocuous stimuli became painful (allodynia)[140]. This peripheral sensitization increases the firing of nociceptors and the intense stimulation induces the release from nerve endings of neuropeptides that can influence a variety of immune cell functions. One of the best-known neuroimmune mechanisms involving sensory neuropeptides is neurogenic inflammation that is involved in arthritis pathogenesis as well as in other inflammatory diseases as psoriasis and Crohn’s disease. Neurogenic inflammation is characterized by a release of neuropeptides by sensory nerves that in turn induces vasodilatation and plasma extravasation, promotes leukocyte chemotaxis and phagocytosis and directly affects the release of inflammatory mediators from mast cells and macrophages [141]. In the mucosae and in lymphoid organs, sensory fibers are in close contact with the cell membranes of mast cells, macrophages, Langherans cells and endothelial cells in what has been defined a “neuroimmune junction”. It is reasonable to hypothesize that, during the inflammatory process and because of tissue damage, there is a release of mediators that modify the local concentration of NGF. Several studies have shown that cytokines involved in inflammation, such as IL-1β, TNF-α and IL-6, are promoters of NGF synthesis in a variety of cell types. Inflammatory cytokines can induce the synthesis of NGF in neuronal and glial cells, as well as in epithelial, endothelial, connective and muscle cells **(Figure 10)** [115]. NGF directly regulates in sensory neurons the expression of neuropeptides such as Substance P (SP) and calcitonin gene related petide (CGRP) that, once released from nerve terminals, induce the synthesis of inflammatory mediators and cytokines in immune cells and affect their functions. While SP has been demonstrated to have a pro-inflammatory role, CGRP, which is often co-localized with SP in nerve endings, can also have an inhibitory action. CGRP is a potent inhibitor of mitogen and antigen-stimulated proliferation of T-cells, antigen presentation by antigen-presenting cells and cytokine synthesis.

Also Systemic Lupus Erythematosus (SLE) patients exhibit a significant increase in NGF concentration in the sera, which correlates with disease activity, and similar findings were obtained in studies of NZB/W mice, a spontaneous animal model of human SLE [142-144].

NGF found at higher levels in the intestines of patients with Inflammatory Bowel Disease IBD (including Crohn’s disease and ulcerative colitis), promotes inflammation and pain in the gut by sensitizing local sensory neurons [145]. In asthma NGF can activate mast cells and other immune cells in the lungs, contributing to chronic airway inflammation and hyperresponsiveness, while in psoriasis NGF influences keratinocyte proliferation and immune cell activity, promoting skin plaques and mediating activation of transient receptor potential vanilloid 1 in neurogenic pruritus of psoriasis[146].It is worth mentioning that the inflammatory diseases characterized by an enhanced production of NGF in the inflamed tissues or in the blood have different pathogenic mechanisms[115].Although some discrepancies exist between the dual effects of NGF *in vitro* and *in vivo*, it is reasonable to hypothesize that NGF is part of an endogenous mechanism that, regulate inflammatory response **(Figure 10).**

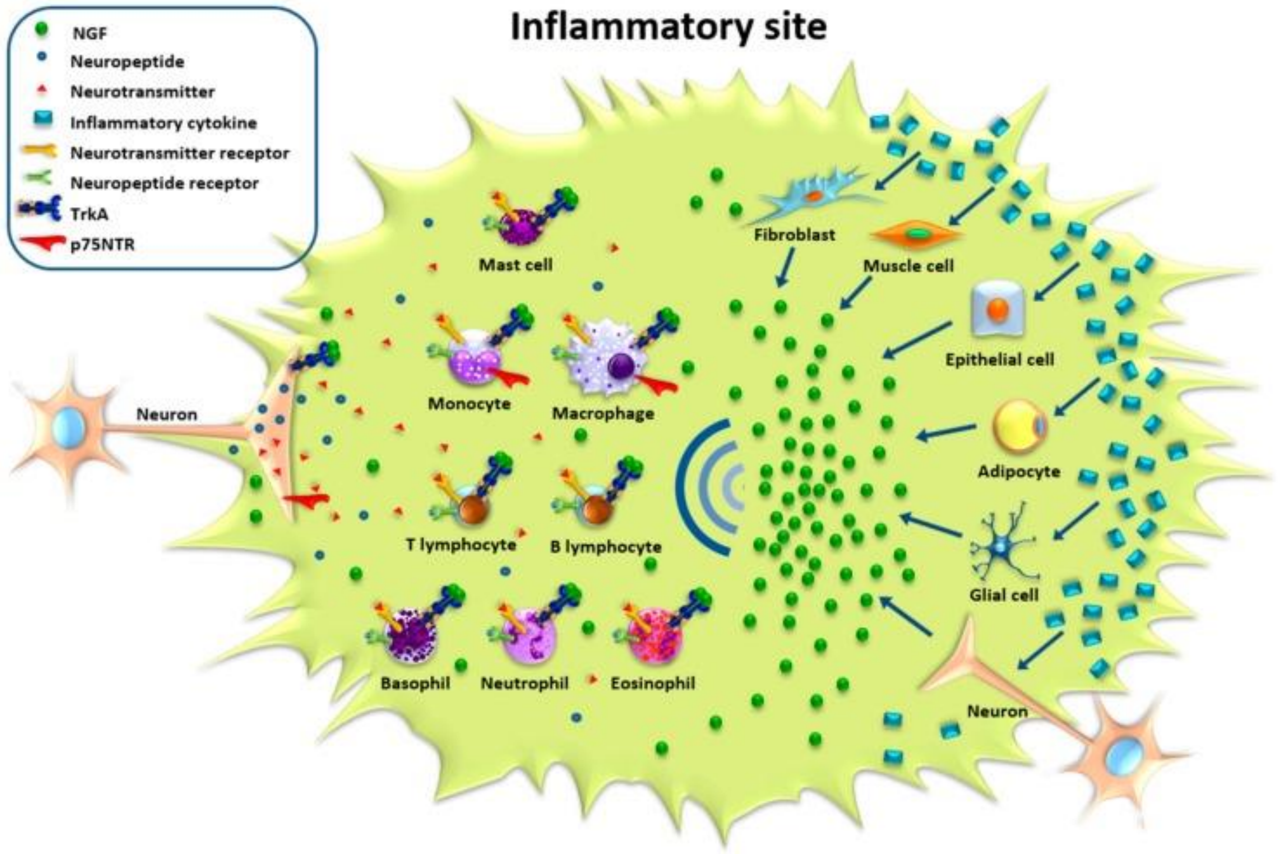

Figure 10. Direct and indirect effects of nerve growth factor (NGF) on inflammatory responses. At the site of inflammation, inflammatory cytokines induce (blue arrows) the production of NGF in different cell types, such as muscle cells, epithelial cells, fibroblasts, adipocytes, neurons, glia, and immune cells. The enhanced local production of NGF influences nerve fiber distribution and neuronal activity, inducing the synthesis and release of neuropeptides and neurotransmitters that have immunomodulatory effects. NGF receptors are also expressed on the membrane of immune cells and NGF can directly modulate the activity and functions of immune cells. [115]

3.2. Rheumatoid arthritis (RA) and juvenile idiopatic arthritis (JIA); clinical features

Among the inflammatory diseases characterized by an involvement of NGF in the inflammatory response, we focus our attention on Rheumatoid arthritis (RA) and juvenile idiopatic arthritis (JIA) which are chronic inflammatory joint diseases leading to a cartilage and bone damage as well as disability. Arthritis typically starts in small peripheral joints, is usually symmetric, and progresses to involve proximal joints if left untreated [147-149]. Joint inflammation over time leads to the destruction of the joint with loss of cartilage and bone erosions. RA with a symptom duration of fewer than six months is defined as early RA and, when the symptoms have been present for more than six months, it is defined as established RA [148]. RA, if untreated, is a progressive disease with morbidity and increased mortality [150]. JIA is the most common chronic inflammatory rheumatic condition of childhood [151] that lasts for >6 weeks with onset before 16 years of age. There is no pathognomonic laboratory test for rheumatoid arthritis, which makes the diagnosis of this disease challenging in the early stages. A comprehensive clinical approach is required to make the diagnosis and prevent debilitating joint damage [148, 149]. The etiology of RA has a significant basis in genetics but the cause of development is still largely unkonwn. Risk factors for RA development, severity, and progression include a strong genetic component. The most important risk is located within the class II major histocompatibility (MHC) locus. HLA-DR4 is found in 70% of RA patients when compared to 30% of control subjects. The pathogenesis of RA might begin at the mucosal level prior to transition toward synovial involvement [152]. Epidemiological studies showed an association between the development of RA and viral and bacterial antigens, smoking, bronchiolar mucosal abnormalities. Autoimmunity starts at the molecular and cellular level long before the clinical phase of arthritis, and it is referred to as pre-symptomatic or pre-clinical arthritis. During the pre-clinical phase, certain immunologic and biochemical abnormalities have been found, but the patients are asymptomatic [153]. Autoantibodies appear before the onset of clinical arthritis [154] and in 80% of patients, rheumatoid factors are presents (antibodies against the FC fragment of IgGM). Many patients develop autoantibodies but do not develop the overt disease. Some patients will eventually transition from autoimmunity to immune-mediated inflammation primarily focused in the synovium [155].These autoantibodies are produced by plasma cells in the synovium. The synovium in patients with arthritis is infiltrated by immune cells, which include innate immune cells (monocytes, dendritic cells, mast cells) and adaptive immune cells (T-helper 1, T-helper 17, B cells, and plasma cells). Neutrophils are not present in the synovium but migrate from the bloodstream to the synovial fluid [156].

All elements of the immune system are involved in the development of the synovial inflammatory. response: innate immunity and adaptive immunity, which includes the cellular (T cell) immune response and the humoral (B cell) immune response [157]. The innate immune response involves macrophages which produce TNF-α, IL-6, IL-1β, GM-CSF, IL-15, IL-18, IL-32, and chemokines, all contributing to promote immune cell infiltration and tissue inflammation in the synovium. [158] **(Figure 7).** Cytokines and chemokines such as tumor necrosis factor-alfa (TNF-α), interleukin-6 (IL-6), and granulocyte-monocyte colony-stimulating factor (GM-CSF) activate endothelial cells and attract immune cells within the synovial compartment. In the inflamed joints, fibroblast-like synoviocytes (FLS) are extremely activated. The FLS in the rheumatoid synovium change to an invasive phenotype producing RANKL which leads to osteoclast generation resulting in bone erosions, the hallmark feature of rheumatoid arthritis [159] It is hypothesized that a second environmental trigger is needed to cause clinically apparent disease. When this is established, a destructive inflammatory process begins. Fibroblast-like synoviocytes (FLS) migrate from joint to joint, leading to progressive joint damage [159]. Resident fibroblasts-like synoviocytes (FLS) are activated by proinflammatory cytokines, PGDF, and chemokines to proliferate with invasive characteristics and produce matrix metalloproteinases (MMP) and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs). Chondrocytes are activated by TNF-α, IL-1β and

IL-17, producing matrix enzymes resulting in cartilage damage. Resident mast cells produce vasoactive amines, leukotrienes, proteases, and TNF-α and, as mentioned above, osteoclasts are activated by RANKL, which plays a critical role in producing bone erosions[156]. No diagnostic criteria exist for arthritis. The typical patient presents tender and swollen joints of recent onset, morning stiffness, and abnormal laboratory tests, such as elevated C-reactive protein (CRP) levels or erythrocyte sedimentation rate (ESR). Unfortunately, these clinical parameters are not at all specific for arthrits patients and there is no pathognomonic finding for arthritis. Early diagnosis and treatment of arthritis can avert or substantially slow progression of joint damage in up to 90% of patients, thereby preventing irreversible disability. Despite treatment, many patients progress to disability and suffer significant morbidity over time. Today, the standard of care is early treatment with disease-modifying anti-rheumatic drugs (DMARDs) that work to decrease pain and inflammation, to reduce or prevent joint damage, and to preserve the structure and function of the joints. Outcomes have been improved by recognizing the benefits of early diagnosis and early therapy with DMARDs. The treatment target is remission or at least a state of low disease activity, which should be attained within 6/9 months. Methotrexate is first-line therapy and should be prescribed in combination with glucocorticoids; 40% to 50% of patients reach remission or at least low disease activity with this regimen. If this treatment fails, sequential application of targeted therapies, such as biologic agents (eg, tumor necrosis factor inhibitors) or Janus kinase inhibitors in combination with methotrexate, have allowed up to 75% of these patients to reach the treatment target over time. New therapies using biological agents have been developed in response to new pathogenetic findings [160]**(Figure 11).**

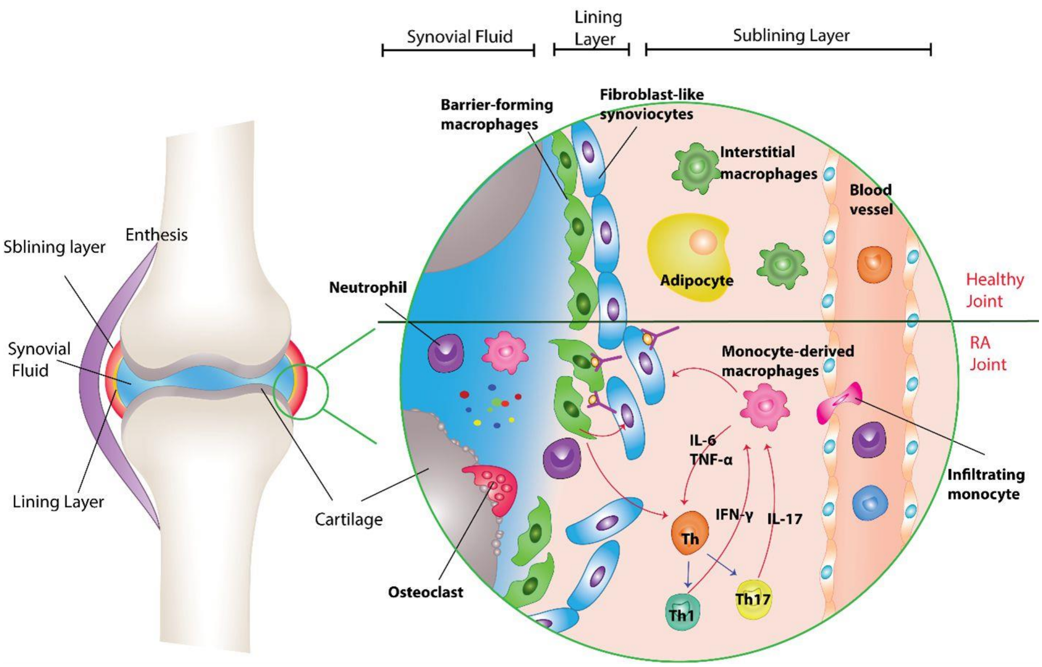

Figura 11. **Rappresentation of inflammed synovia in arthritis patients.** The normal synovial membranes covering the inner side of synovial joints are thin and composed of the lining and sublining layers. Interstitial macrophages are derived from non-monocyte lineage present in the sublining layer of the synovial membrane. They are differentiated into barrier-forming macrophages and along with synovial fibroblasts form the lining layer. The barrier-forming macrophages, protect the synovium from inflammatory attacks by reducing the infiltration of immune cells through tight junctions, suppressing inflammation, and digesting infiltrated neutrophils. IFN-γ and IL-17 secreted from Th 1 and Th17 are the cytokines responsible

for the stimulation of macrophages to release other cytokines like IL-6, and TNF-α, which leads to the initiation of the inflammation process. These cytokines released from the macrophages, in turn, activate the differentiation of T cell lymphocytes into Th1 and Th17 subtypes. It is found that macrophages can also regulate FLS function and behavior in a feedback loop.

4. Activation of proNGF/p75NTR axis as a novel inflammatory mechanism in arthritis

4.1 The imbalance between NGF/proNGF forms in pathological conditions

In addition to the known effects of NGF on inflammatory pain and neurogenic inflammation in chronic arthritis, NGF could also be directly involved in regulating the inflammatory process. A recent study in our lab demonstrated that a marked down-regulation of TrkA expression characterises mononuclear cells of patients with JIA, who showed an altered NGF regulatory feedback mechanism: in contrast to observations in healthy donors, the addition of NGF did not reduce interleukin (IL)-6 production from JIA mononuclear cells (MNCs) (prencipe et al 2014). To assess what kind of anomalies occour in the NGF pathways in patients with JIA, we find an altered balance between the NGF receptors TrkA and p75NTR with a higher expression of p75NTR and a concomitant decrease in TrkA at both the mRNA and protein levels, in mononuclear cells obtained from blood and synovial fluids of JIA patients. On the contrary, healthy donors show a completely different ratio with TrkA as the prevalent receptor expressed in mononuclear cells. Associated to the high expression of p75NTR in JIA patients, we found that there is a strong up-regulation of its co-receptor sortilin, a transmembrane receptor belonging to the Vps10p domain-receptor family that specifically increase the affinity of p75NTR to its ligand proNGF, the immature form of NGF. Together with the high expression of p75NTR in MNCs in local inflamed synovia, we found that proNGF and not mNGF is the prevalent form detected in synovial fluids of patients with JIA and RA with synovial fibroblasts as a major source of proNGF in the inflamed synoviae [161].

This observation was possible thanks to a novel developed ELISA able to discriminate between mature and immature form of NGF while in the past the analysis of NGF levels could not distinguish between proNGF and NGF. Therefore, the reported increase in NGF concentration found in inflamed tissues or biological fluids actually represents a total amount of the two forms of NGF while at the time it was believed to represent the increase in the concentration of mature NGF only.

Very little is known about the relative concentrations of proNGF and mature NGF in inflammatory diseases and the specific effects of proNGF and mature NGF on inflammatory responses are not yet clear. Our *in vitro* data showing that binding of proNGF to its specific p75NTR enhances inflammatory cytokine production and the inhibition of p75NTR activity in JIA MNCs and synovial fluids mononuclear cells (SFMCs) reduces cytokine production [161]. proNGF and p75NTR activate a completely different biological response from mNGF which binds preferentially TrkA receptors activating pro-survival pathway, differentiation, and tissue repair instead. Moreover, recent evidence in literature highlighted the role of proNGF in different inflammatory conditions: in the cerebrospinal fluid (CSF) proNGF represents a promising diagnostic biomarker for

Alzheimer Disease (AD) and it was demonstrated that an increase in proNGF level was associated with neurodegeneration in early AD and that proNGF/NGF ratio is an upstream driver for neurodegeneration both in animal models and in humans [162]. The altered balance between proNGF and mature NGF influences neuroinflammation and repair processes in Multiple Sclerosis (MS), with proNGF contributing to tissue damage and inflammation. Indeed, proNGF induces p75-Mediated death of oligodendrocytes following Spinal Cord Injury [85]. Increased levels of proNGF have been detected in the inflamed intestinal tissues of patients with IBD, including Crohn's disease and ulcerative colitis, promoting inflammation through immune cell activation and cytokine release, which leads to further mucosal damage and dysregulation of the gut barrier. In IBD, proNGF may also influence pain perception by sensitizing visceral nociceptors, contributing to the abdominal pain often associated with the disease. proNGF is upregulated in patients with chronic asthma, where it contributes to the activation of mast cells and promotes the release of histamines and other inflammatory mediators, worsening airway hyperresponsiveness and chronic inflammation [163]. proNGF levels contribute to retinal inflammation and neuronal cell death in diabetic retinopathy, promoting the production of inflammatory mediators like vascular endothelial growth factor (VEGF), which leads to vascular leakage and further retinal damage. The pro-apoptotic effects of proNGF through p75NTR signaling exacerbate retinal neurodegeneration, which is a hallmark of advanced diabetic retinopathy [164].

5. Specific aims:

This project is based on the hypothesis that the modified balance of the expression of the NGF receptors, TrkA and p75NTR, and proNGF/NGF relative concentrations are key points of regulation of the inflammatory response in chronic arthritis. Our working hypothesis is that an abnormal p75NTR expression, associated with high proNGF concentration in the inflamed tissue, leads to the continuous activation of the proNGF/p75NTR pathway, increasing the release of inflammatory mediators, thus contributing to chronic joint inflammation.

In order to demonstrate that proNGF/p75NTR axis could be a novel target for the treatment of chronic arthritis we investigated whether:

- abnormalities in TrkA and p75NTR expression in peripheral blood cells and synoviae of patients with RA and JIA are associated with disease progression;

- the expression and activation of p75NTR in synovial fibroblasts can be induced *in vitro* by inflammatory stimuli;

-the inhibition of p75NTR pathways can modify the inflammatory response of synovial fibroblasts;

- inhibition of proNGF/p75NTR in an animal model of antigen induced arthritis (AIA) could influence the clinical signs of the disease and affect immune cells functions;

- p75NTR-ECD levels measured in blood and synovial fluids of RA and JIA patients is associated with clinical feature es of arthritis and could represent a novel diagnostic biomarkers;

The ultimate goal of this project is to generate a comprehensive body of evidence providing the rationale for the use of p75NTR antagonists in the treatment of chronic arthritis in humans.

6. Materials and Methods:

The experimental work has been performed using cells and biological fluids from JIA and RA patients for *ex vivo, in vitro,* and *in vivo* experiments using a murine model of antigen-induced arthritis (AIA).

Ex vivo analysis and in vitro experiments:

Patients:

Synovial fluids (SF) were obtained from patients with juvenile idiopathic arthritis (JIA) followed at the Division of Rheumathology of Bambino Gesú Children's Hospital. The study was approved by the local ethical committee (ID#2333_OPBG_2020) and written consent was obtained from parents of children, as appropriate. Total of 8 synovial tissues were obtained from treatment-naïve (n=4) and in sustained clinical and ultrasound remission (n=4) RA patients followed at the SYNGem Biopsy Unit of the Fondazione Policlinico Universitario A. Gemelli IRCCS, as approved by the Institutional Ethics Committee (ID#6334/15). The patients involved provided signed informed consent. Diagnosis of JIA and RA were based on the International League of Associations for Rheumatology classification criteria and on 2010 EULAR/ACR criteria [165]. JIA patients (n= 18) followed at the Bambino Gesu' Children Hospital had oligoarticular, extended oligoarticular, or polyarticular JIA. Ten were females. The median age at disease at sampling was 9.4 years (IQR 2.1–12.7) and the median disease duration at sampling was 2.8 years (IQR 1.9–8.7). All patients had active disease with overt arthritis at sampling. 11 patients were untreated, 7 were receiving traditional DMARDs. Patients receiving glucocorticoids were not included. Blood samples were collected from fasting participants and then centrifuged at 2000 rcf at 4 °C for 10 min. Serum was separated, and aliquots were stored at −80 °C until assayed with different ELISA tests.

Single Cell-RNA Sequencing of RA Synovial Tissue

Methods for single-cell RNA sequencing (scRNAseq) of synovial tissue have already been described in detail [166]. Briefly, synovial tissue biopsies were collected from naive to treatment RA patients (n=4) and from RA patients in sustained clinical and ultrasound remission (n=4) at the SYNGem Biopsy Unit of the Fondazione Policlinico Universitario A. Gemelli IRCCS using US-guided minimally invasive technique [167]. High-quality total RNAs (RIN >8) were used to construct Illumina mRNA sequencing libraries. cDNA synthesis and amplification were performed by using SMART-seq v4 Ultra Low Input RNA Kit for Sequencing (cat. no. 634890, Takara) starting with 10 ng of total RNA, following the manufacturers protocol. 10 ng of amplified cDNAs were sheared prior to preparing the final libraries using the Bioruptor® Pico system (Diagenode, 24 cycles of 30 sec on and 30 sec off). Dual indexed Illumina sequencing libraries were prepared by SMARTer® ThruPLEX® DNA-seq 48D Kit (cat. no. R400406, Takara) following the kit protocol. The pooled libraries were sequenced at Glasgow Polyomics (Glasgow, UK) on a NovaSeq 6000 system using a read length of 100 bases in paired-end mode. The reads were mapped with STAR (version 020201) with default parameter against the Human genome version GRCh38, release 91. The read count matrix was constructed with featureCounts (Version 1.6.4) using default parameters. All differential expression analysis was performed in R using the DESeq2 package. All genes with an adjusted p value < 0.05 and a log fold change of > +/- 1,5 were considered significantly differentially expressed. All raw and processed data were deposited at EMBL-EBI and are available with the accession number E-MTAB-8322.

Fibroblast like synoviocytes culture

FLS from rheumatoid arthritis patients (RA-FLS) (n=8) and osteoarthritis patients (OA-FLS) (n=6) were purchased from CliniSciences (Abbiotec, Escondido, USA). Control skin fibroblasts (CTRL) were from American Type Culture Collection (ATCC). Cells were cultured in 10% fetal bovine serum (FBS) high-glucose Dulbecco's Modified Eagle Medium (complete DMEM) with or without the addition of different doses of human recombinant IL1-β or TNF-α (all from R&D systems, Minneapolis, MN), or LPS (Sigma Aldrich, St Louis, MO). 200 ng/ml of proNGF cleavage-resistant which has an R-to-G substitution at amino acid position 104 (Alomone Labs, Jerusalem, Israel) were used to stimulate cells cultured in AIMV Serum Free Medium (Life Technologies, Rockville, Maryland, USA). Supernatants were collected after 18 hours of incubationin order to evaluate the production and the release of IL-6 . *Ex vivo* experiments were performed with RA-FLS cultured in complete DMEM with or without the addition of 30% v/v synovial fluid. Cells were pre-treated 1 hour at 37°C with or without LM11A-31 (a kind gift of Dr. Frank Longo, Stanford University) or with anti-p75NTR antibodies (clone ME20.4 Millipore, Billerica, MA, USA) to test p75NTR inhibition or the anti-IL-1β monoclonal antibody canakinumab (5 μg/ml).

Immunofluorescence Analysis

RA-FLS and OA-FLS were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 PBS, incubated for 1 hour at RT with 1% BSA, 5% goat serum (Abcam, Cambridge, UK) PBS and then with mouse anti-p75NTR antibody (Merck, Darmstadt, Germany, clone 8211) or rabbit anti-proNGF (Merck AB9040) and Alexa Fluor secondary antibodies (Invitrogen). Confocal imaging acquisition was performed on Olympus Fluoview FV1000 confocal microscope using a 40× (0.90 NA oil) objective

RNA Extraction and Real-Time PCR Analysis

After RNA extraction using Trizol Reagent (Thermo Fisher Scientific, MA), cDNAs were retro-transcribed using the Superscript Vilo kit (Invitrogen, CA). Real-time PCRs were performed using TaqMan Universal PCR Master Mix and gene expression assays from Applied Biosystems (CA, USA). TrkA, p75NTR, NGF, sortilin mRNA expressions were tested using Assays on Demand reagents (TrkA Hs01021011_m1; p75NTR Hs00182120_m1; NGF Hs00171458_m1; sortilin Hs00361760_m1). TaqMan Endogenous Control human HPRT Hs 02800695_m1 and GAPDH Hs 99999905_m1 (Applied Biosystems) were used as housekeeping genes. Normalized gene expression levels were calculated as 2-ΔCt [ΔCt = Ct (gene of interest) – Ct (housekeeping gene)] and results were expressed in arbitrary units (A.U.). Fold changes were calculated using the 2-ΔΔCt equation [ΔΔCt = ΔCt (treated sample) – ΔCt (untreated sample)][168].

Cytokine and NGF-proNGF ELISA

Levels of p75NTR-ECD, IL-6, MCP1, IL-8 were analyzed using R&D Quantikine ELISA. Conditioned media and SF were assayed using ELISAs specific for human mature NGF or proNGF, as described [22].

Western Blot Analysis

After RIPA buffer lysis (Cell Signaling, Leiden, The Netherlands), protein concentration was measured with BCA Protein assay (Thermo Fisher Scientific). 30 µg protein extracts were resolved by 10% SDS PAGE, transferred to nitrocellulose membranes and probed with antibodies against: phospho-stress-activated protein kinase/c-Jun NH(2)-terminal kinase (SAPK/JNK) (Thr183/Tyr185, clone G9 MAB #9255), total SAPK/JNK (#9252), phospho-p38 Mitogen-Activated Protein Kinase (MAPK) (Thr180/Tyr182, clone 28B10 MAB #9216), total p38 MAPK (#9212) (all from Cell Signaling), p75NTR (clone 8211 MAB5264) (EMD Millipore), GAPDH (clone G9, MAB #32233) (Santa Cruz Biotechnology), and tubulin (Sigma Aldrich). Blots were developed by ECL system (Amersham Biosciences) according to the manufacturer's protocol.

Apoptosis Detection

Cells were incubated with or without LM11A-31 (10, 100 nM) for 1 hour at 37°C and then 30% v/v SF was added. After 18 hours apoptotic cells were stained using Annexin V-FITC Apoptosis Detection Kit (Sigma Aldrich). Cells were analyzed by flow cytometry (FACSCanto II, BD Biosciences CA, USA).

4.2 In vivo experiments:

Mouse AIA induction and treatment

Animal experimentations in this study were performed according to the guidelines of the Animal Experimentation and following the institutional guidelines (8972020PR A69A0). Male C57BL/6 mice were purchased from Charles River and maintained in pathogen-free conditions at Plaisant s.r.l. animal facilities. To induce AIA, mice were immunized intradermally with 100 µg of methylated BSA (mBSA) (Sigma-Aldrich, St Louis, MO, USA) emulsified with an equal volume of complete Freund's adjuvant (Sigma Aldrich)[169]. They were injected with 2 × 109 Bordetella pertussis (Kaken Pharmaceutical, Tokyo) i.p. This procedure was repeated after one week and after three weeks (DAY 0). 100 µg of mBSA in 10 µl of saline was injected into the right knee joint (AIA group). Control mice were not immunized with mBSA and complete Freud's adjuvant, but were injected with saline into left knee joint at day 0 (CTRL). While, a third group, AIA-treated mice received a specific p75NTR inhibitor, LM11A-31, by orally gavage from day 0 to day 7. All mice were sacrified at day 7.

Tissue preparation for histology and histological stainings

For proper processing of knee joints for histology, hind paws were excised above the ankle and the skin was removed to allow penetration of formalin. Paws were immediately fixed in 10% formalin for 24 hours. Fixed samples were then decalcified in 14% EDTA solution in gentle shaking condition for 1-4 weeks. The decalcification solution was weekly replaced. Decalcified samples were then paraffin-embedded and cut on a regular microtome at a thickness of 2-5 µm. The bone sections were deparaffinized, re-hydrated and stained for the following standard histological stainings: Hematoxylin and Eosin (H&E) staining for quantification of synovial inflammation and TRAP staining for detection of multinucleated osteoclasts and scoring the extent of bone and cartilage erosions. Moreover, an immune-histochemistry for p75NTR detection was performed after proteinase K antigen retrieval. Synovial inflammation was then arbitrary scored ranging from 0 (healthy) to 3 (severe) with in-between grading scores of 0.5, according to guidelines reported in Hayer S, et al. Ann Rheum Dis 2021. Histomorphometric measurements were performed on sections with an interactive image analysis system (NIS-Elements BR 4.50.00; Tokyo, Japan). Nomenclature, symbols, and units of histomorphometric bone parameters were those suggested by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research [170].

Immunohystochemistry in spleen sections

Sections (7 µm in thickness) were cut from paraffin blocks, mounted on slides and stored at room temperature. The sections were stained with hematoxylin and eosin (H&E) and examined using a Olympus Fluoview FV1000 confocal microscope for general morphological characterization and to highlight the presence or absence of structural alterations. Other sections obtained from the same animals were also stained in order to evaluate the p75NTR distribution. Antigen retrieval was performed by heating the sections in 10 mmol/L citrate buffer (pH 6.0) for 5 minutes. Rabbit polyclonal anti-p75NTR antibody, (Cell Signaling, #8238), was used as primary antibody at a 1:1000 dilution.

Immunofluorescence and image analysis

Frozen spleens sections were cut using a cryostat and sections (7 µm in thickness) were permeabilized with 0.1% Triton X-100 in PBS 1X for 10 min at RT and blocked with 10% normal goat serum (NGS) (Merck) in PBS 1X (blocking solution) for 1 hour. Sections were incubated with rabbit anti-MARCO (Abcam) and rat anti-B220 (Invitrogen) primary antibodies in blocking solution overnight at 4 °C. After three washes with PBS 1X, the slices were incubated with anti-rabbit/AF488 and anti-rat/AF647 (Invitrogen) secondary antibodies in the blocking buffer solution for 1 h and, after, stained with Hoechst for nuclear detection. All images used for quantification of the immunofluorescence were acquired with an Olympus confocal microscopy. Images were obtained using a 63x oil objective at a resolution of 1024 × 1024 pixels. For each set of experiments, all images were acquired using identical exposure settings. Offline analysis was performed using the ImageJ software. Follicular morphology was quantified by using the “Polygon Selections” feature tool and the “Measure” function of ImageJ yielding the following shape descriptors: field area (μm2) and field perimeter (μm).

Flow cytometry analysis of mouse splenocytes

In order to evaluate p75NTR expression on the splenocyte subpopulations, single-cell suspensions were prepared from spleen of mice in different groups on the termination day (day 7). Cells were stained with anti-CD45/APC, anti-B220/BV510, anti-CD3/BB700, anti-Ly6C/BV421, anti-CD11b/FITC, anti-CD335/BUV395, anti-CD11c/BUV805 and anti-CD271/PE mAbs for 20 min at 4°C. In order to evaluate Th17/Treg ratio, splenocytes were stained with anti-CD45/BUV395, anti-CD3/BV786, anti-CD4/Pecy7, anti-CD271/PE for 20 min at 4°C. For intracellular staining, cells were fixed and permeabilized with the Foxp3/Transcription factor Staining kit (Thermo Fisher, Waltham, MA, USA), according to manufacturer’s instructions and incubated for 20 min at 4°C with anti-Foxp3/APC and anti-Rort/PECF594 mAbs. Post incubation, samples were washed once with PBS 1X and analyzed by FACS LRSFortessa (BD Biosciences, San Jose, CA, USA). Flow cytometric analysis was performed using FlowJo Flow Cytometric Analysis Software.

Quantitative Real Time PCR of mouse tissues

Cartilage specimens were washed thoroughly with sterile PBS to remove blood and to identify areas of full-thickness articular cartilage to be harvested[171]. Cartilage were snap frozen in liquid nitrogen to proceed to RNA extraction. TRIzol® (Thermo Fisher Scientific, MA) was thawed, and mixed thoroughly with a 1 ml pipette to dissolve all powdered tissue in the flask. After RNA extraction using TRIzol® Reagent protocol following manufacturing instrucitons, cDNAs were retro-transcribed using the Superscript Vilo kit (Invitrogen, CA). Real-time PCRs were performed using TaqMan Universal PCR Master Mix and gene expression assays from Applied Biosystems (CA, USA). Il-6, Il-1β, Tnf, Ccl-2 mRNA expression were analyzed (Il-6: Mm00446190_m1, Il-1β Mm00434228_m1, Tnf Mm00441883_g1). TaqMan Endogenous Control Gapdh (Mm99999915_g1) were used as housekeeping genes. Normalized gene expression levels were calculated as 2-ΔCt [ΔCt = Ct (gene of interest) – Ct (housekeeping gene)] and results were expressed in arbitrary units (A.U.). Fold changes were calculated using the 2-ΔΔCt equation [ΔΔCt = ΔCt (treated sample) – ΔCt (untreated sample)] [168].

ELISA Assay for Antibody Determination

ELISA assay was used to determine serum levels of total and mBSA-specific IgG. Briefly, 96-well ELISA plate was coated with 50 ng/mL of anti-Mouse IgG-IgA-IgM primary antibody (Sigma) or 25 ug/mL mBSA (Sigma) overnight at 4°C, washed with PBS 0.05% Tween-20 and incubated with PBS/ 1%BSA for 2 h at RT. After three washes, 100 ul of samples were added overnight at 4°C and then incubated with hrp-linked goat anti-mouse IgG secondary antibody (Invitrogen) for 2 h at RT. 50 µL of TMB solution were added, and the OD value was measured at $\lambda = 450$ nm after 10 min at RT.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 5 Software (GraphPad Software, La Jolla, CA). Statistical significance is shown as *$p<0.05$ **$p<0.01$ and ***$p<0.001$.

7.Results:

7.1 *Ex vivo:* p75NTR expression is markedly increased in distinct synovial fibroblasts clusters from RA patients

To determine which immune or stromal cells might be activated by the proNGF/p75NTR axis in synovial tissue, we investigated the expression of p75NTR, TrkA and SORT1 using scRNAseq analysis in RA tissue referring to whole synovial tissues (total 118.622 cells) derived from treatment-naïve RA patients or from RA in sustained clinical and imaging remission **(Figure 12A)** [166]. We found that at among different synovial fibroblast clusters, in treatment-naïve RA synovial tissues, lining layer PRG4pos cluster and sublining THY1posCOL1A1pos fibroblast cluster are enriched of p75NTR, NGF and sortilin expression compared to other FLS clusters as well as to other resident and inflammatory synovial cells such as macrophages or T and B lymphocytes **(Figure 12B)**. The expression of p75NTR characterizes other resident and inflammatory synovial cells such as macrophages or T and B lymphocytes that drive inflammation and damage [172]. To determine which immune or stromal cells might be activated

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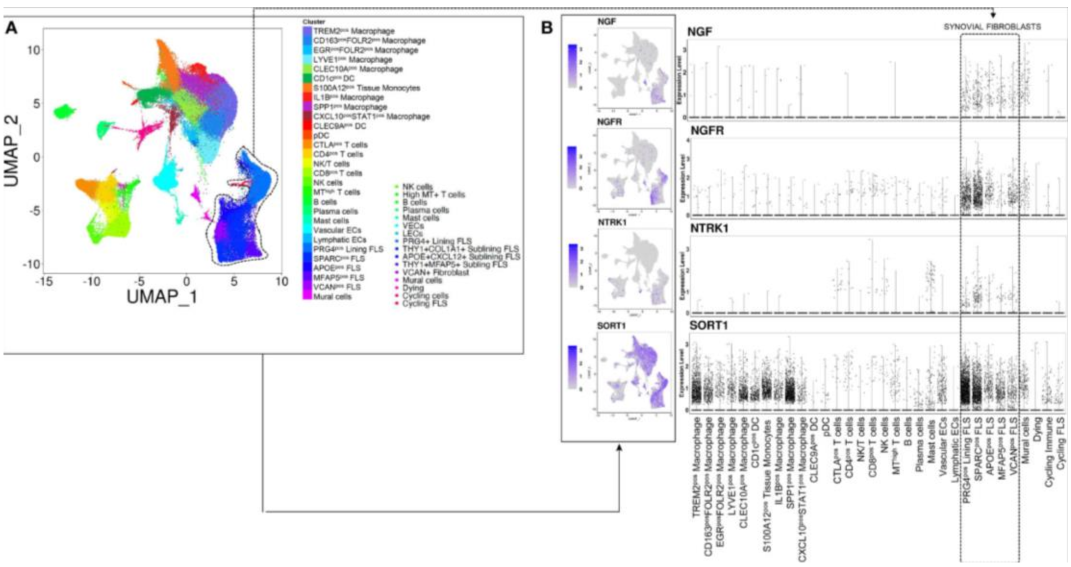

Figure 12. NGF, p75NTR, TRKA and SORT1 are expressed in lining and sublining synovial fibroblasts clusters from treatment-naïve patients with rheumatoid arthritis. A) UMAP (Uniform Manifold Approximation and Projection for Dimension Reduction) of synovial tissue cells from treatment-naïve Rheumatoid Arthritis patients (n = 4) (27) by scRNAseq. Each dot represents a cell and distinct cell clusters are identified according to the color legend. B) UMAP and dot plots showing NGF, NGFR, NTRK1 and SORT1 gene expression in distinct inflammatory and stromal cells clusters. The violet color identifies cells expressing NGF, p75NTR, TrkA and sortilin (Farina et al., 2022)

In RA patients in sustained clinical and imaging remission, scRNAseq analysis revealed that p75NTR and TrkA expression is significantly reduced in THY1posCOL1A1pos fibroblast cluster when compared to treatment-naïve RA **(Figure 13).** We also observed a significant reduction of SORT1 expression in the lining layer (PRG4pos cluster) as well as in sublining layer fibroblasts (THY1posCOL1A1pos). These data support the notion that proNGF/p75NTR axis is actively involved in RA synovial inflammation, being significantly repressed once sustained remission is achieved.

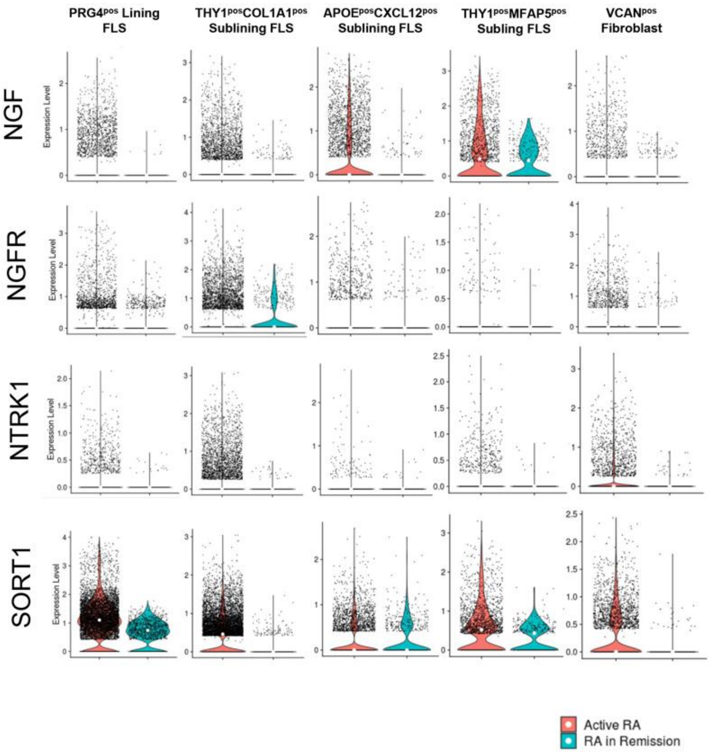

Figure 13. Violin plots showing NGF, NGFR, NTRK1 and SORT1 gene expression in distinct stromal cells clusters from synovial tissue of naive to treatment RA (n=4) and RA in sustained clinical and ultrasound remission (n=4). Data are reported as mean (white dot) [173]

7.2 Expression of p75NTR and proNGF release by RA Synovial Fibroblasts

Given the high *in vivo* expression of p75NTR in pathogenic synovial fibroblast clusters in RA synovium, we investigated whether the high p75NTR expression is mantained in RA-FLS i*n vitro* and what could be the biological effect of an active p75NTR pathway in these cells. We analyzed p75NTR and its co-receptor sortilin expression also in osteoarthtis (OA) OA-FLS used as a pathological control. OA is a degenerative non-autoimmune disease characterized by some inflammation of the joints due to degenerative joint impairment. To verify the basal expression of p75NTR in non-inflamed conditions we used skin fibroblasts (skin-FB) from healthy donors. Our data indicate that p75NTR is expressed far more in RA-FLS than in either OA-FLS or skin-FB **(Figure 14A)**, while control skin fibroblast express higher levels of TrkA than RA-FLS. Thus while in control fibroblasts the main NGF receptor expressed is TrkA, in RA FLS p75NTR is the predominant receptor, indicating a complete inverted balance between the two NGF receptors.

p75NTR mRNA expression data were confirmed by Western blot **(Figure 14B)** . *In vitro* stimulation with IL-1β marginally up-regulated p75NTR mRNA expression in OA-FLS **(Figure 14C)**, while induced a marked increase in RA-FLS **(Figure 14C)**. Immunofluorescence confirmed a higher basal expression of p75NTR and its further enhancement after stimulation with IL-1β in RA-FLS, while only a slight increase was observed in OA-FLS using a maximal dose (10ng/ml) of IL-1β **(Figure 14D)**. Other pro-inflammatory stimuli, such as TNF-α and LPS, also upregulate p75NTR expression in RA-FLS indicating a close relationship between inflammation and the overexpression of p75NTR in this biological system **(Figure 14E)** [173]

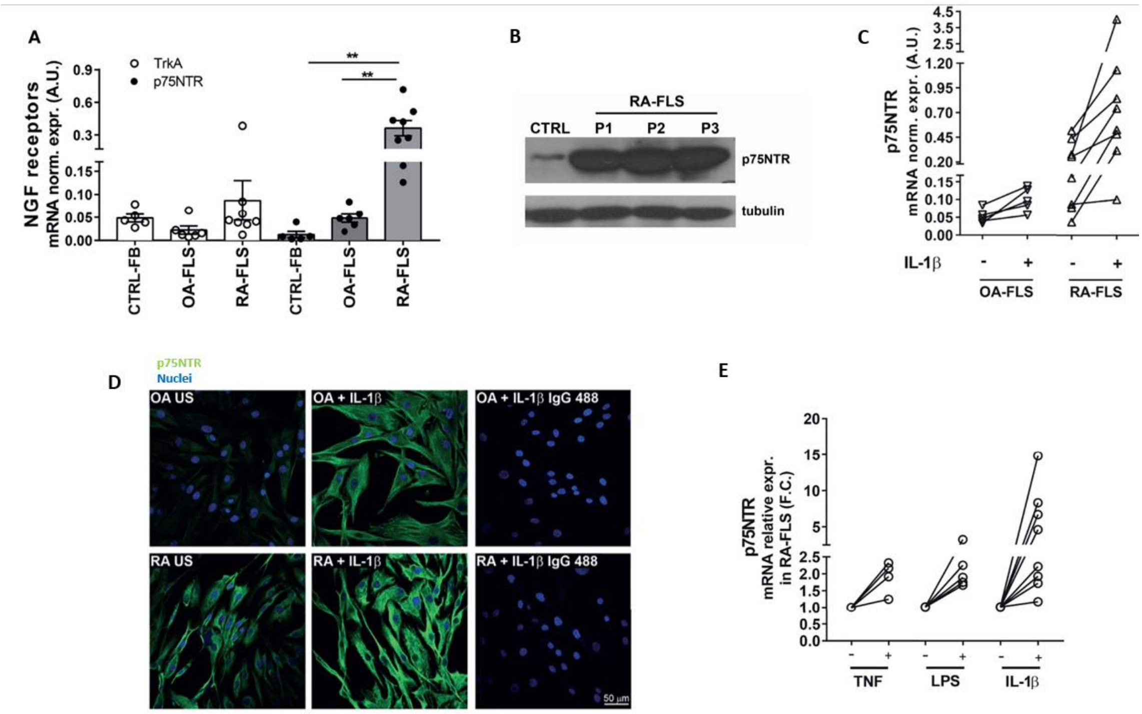

Figure 14. **NGF receptor levels in synovial fibroblasts from RA patients**. A) FLS from RA patients (RA-FLS) (n = 8) have lower TrkA and higher p75NTR mRNA expression levels than OA-FLS (n = 6), and skin fibroblasts (CTRL-FB) (n = 5). Results are reported in arbitrary units (A.U.) after normalization with HPRT expression. Data were analyzed using unpaired t-test (**$p < 0.01$). B) Western Blot analysis of p75NTR protein expression in RA-FLS compared with CTRL-FB. C) p75NTR is upregulated after 1 ng/ml IL-1β stimolation (n = 8) compared to OA-FLS (n = 5). Results are reported in arbitrary units (A.U.) after normalization with HPRT expression. D) Immunofluorescence analysis confirms higher basal levels of p75NTR in RA-FLS (Untreated, RA-US) than in OA-FLS (OA US), while addition of IL-1β (10 ng/ml) further enhances p75NTR in RA-FLS. Representative images of three independent experiments are shown. p75NTR immunofluorescence is shown in green, nuclei in dark blue while anti-Ms IgG 488 is used as negative control for both OA and RA sections. Scale bar 50mm. E) Inflammatory stimuli, 100 ng/ml TNF-a (n = 4), 100 ng/ml LPS (n = 5), and 1 ng/ml IL-1β (n = 8), induce p75NTR expression in RA-FLS. Results are compared to unstimulated condition and calculated as Fold Change (F.C.) [173]

Unstimulated RA-FLS express higher basal levels of NGF mRNA if compared with OA-FLS and CTRL-FB. In RA-FLS the already high NGF mRNA levels were further increased after stimulation with IL-1β **(Figure 15A)** as well as by TNF-α or LPS **(Figure 15B)**. As shown in **Figure 15C** proNGF and not mNGF is the prevalent NGF form released by RA-FLS, confirming what previously measured in the synovial fluids of JIA and RA patients [161]. IL-1β further enhances *in vitro* the secretion of proNGF in RA-FLS, as well as other inflammatory mediators such as LPS or

TNF-α **(Figure 15C)**. Immunofluorescence showed that there is a higher basal expression of proNGF in unstimulated RA-FLS that is markedly increased after IL-1β stimulation **(Figure 15D)**[173]. In this context, we found that, among different inflammatory stimuli, IL-1β is the most effective one enhancing both the expression of p75NTR in RA-FLS and the production of large amounts of proNGF, but not of mature NGF.

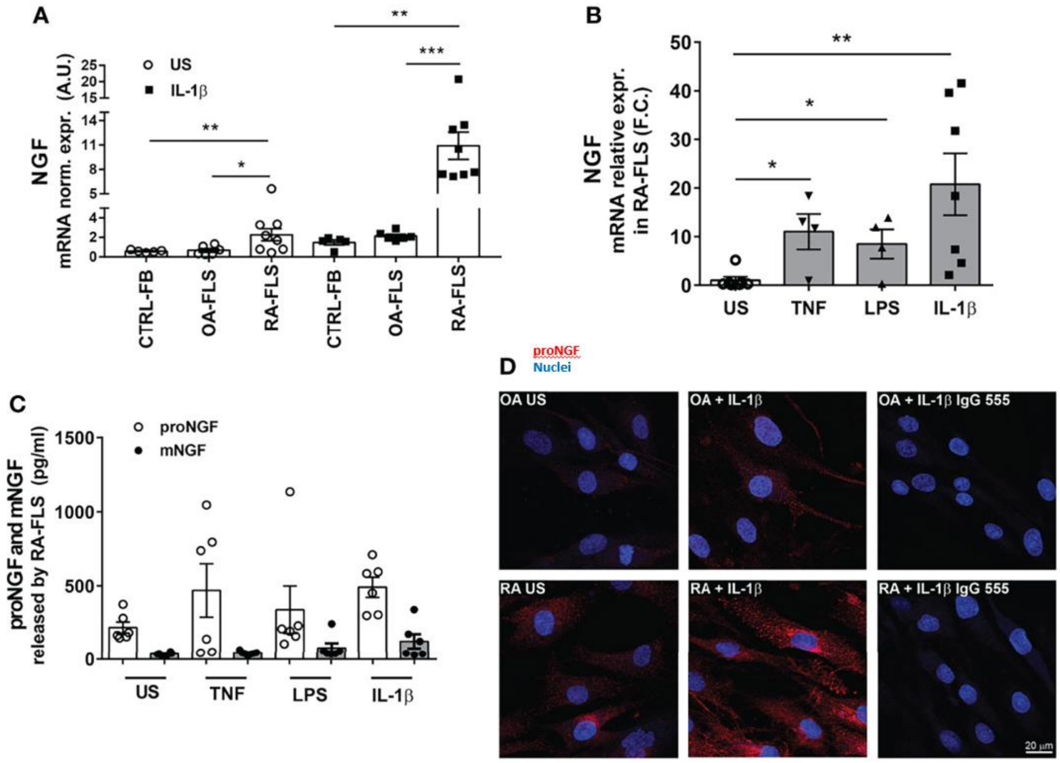

Figure 15. **Inflammatory stimuli enhance NGF expression in synovial fibroblasts from RA patients.** A) Stimulation with 1 ng/ml of IL-1β induces the expression of NGF in RA-FLS (n = 8), OA-FLS (n = 6) and skin-fibroblasts (CTRL-FB) (n = 5). Results are expressed as arbitrary units (A.U.) after normalization with HPRT. Data were analyzed using unpaired t-test (**p < 0.01). B) Inflammatory stimuli enhance NGF mRNA expression in RA-FLS. Basal levels of NGF expression were measured in unstimulated (US) RA-FLS (n = 7) and in RA-FLS stimulated with 100 ng/ml TNF-α (n = 4), 100 ng/ml LPS (n = 4) and 1 ng/ml IL1-β (n = 7). Results are compared to unstimulated condition and calculated as Fold Change (F.C.). Data were analyzed using unpaired t-test (*p < 0.05, **p < 0.01, ***p < 0.0001). C) Newly developed ELISAs that discriminate between proNGF and mNGF showed that proNGF is the most abundant form in conditioned media collected from RA-FLS. proNGF production is enhanced by inflammatory stimuli (100ng/ml TNF, 100 ng/ml LPS, 1 ng/ml IL-1β) in RA FLS (n = 6). D) RA FLS show an increase in proNGF basal protein synthesis after stimulation with IL-1β (1 ng/ml). A lower immunofluorescence for proNGF was observed in OA-FLS after IL-1β stimulation. Representative images of three independent experiments are shown. proNGF immunofluorescence is shown in red, nuclear staining in dark blue, while anti-Rb IgG 555 is used as negative control for both OA and RA sections. Scale bar 50μm. [173]

7.3 p75NTR activation enhances inflammatory cytokine production by RA Synovial Fibroblasts

To understand the functional relevance of proNGF-p75NTR interaction, we stimulated RA-FLS with recombinant proNGF and analyzed the production of inflammatory cytokines using a serum-free medium to avoid interference by NGF and/or proNGF present in fetal bovine serum (FBS) [174]. The addition to RA-FLS of proNGF alone did not modify the release of IL-6 **(Figure 16A)**. In contrast, the addition of proNGF together with sub-optimal concentrations of IL-1β significantly enhanced IL-6 release **(Figure 16A).** The synergy between IL-1β and proNGF was lost at higher IL-1β concentrations. This lack of effect of the exogenous proNGF addition at maximal IL-1β concentrations could be explained by the presence of an autocrine loop since IL-1β induces an increase in both p75NTR and proNGF expression in RA-FLS **(Figure 16A, Figure 15C, Figure 14C)**. When exogenous pro-NGF was added to IL-1β activated RA-FLS, the LM11A-31 blocking of proNGF binding to p75NTR, resulted in a more pronounced inhibition of IL-6 release than would be expected if only exogenous proNGF binding was blocked **(Figure 16A)**. To interpret this data, it should be considered that LM11A-31 prevents not only the binding to p75NTR of the exogenously added proNGF but also the binding of the endogenously-produced proNGF, whose synthesis is strongly activated in RA-FLS by IL-1β stimulation. Indeed, in IL-1β-activated RA-FLS in the absence of exogenously-added proNGF, the blocking of the binding of the endogenously-produced proNGF to p75NTR resulted in a marked decrease in inflammatory cytokine production, indicating the functional interaction of p75NTR and proNGF and the presence of a pro-inflammatory autocrine loop **(Figure 16A)**. Consistently with an autocrine loop, in RA-FLS stimulated with IL-1β, TNF-α or LPS without the addition of exogenous proNGF, the selective blocking of the binding site for proNGF on p75NTR, using a small non-peptide p75NTR ligand inhibitor (LM11A-31) [175] resulted in a significant decrease in inflammatory cytokine production **(Figures 16B–D)**. Similarly, the blocking of p75NTR using a p75NTR-neutralizing antibody significantly decreased IL-6 production in RA-FLS stimulated with different inflammatory stimuli **(Figures 16E)** [173].

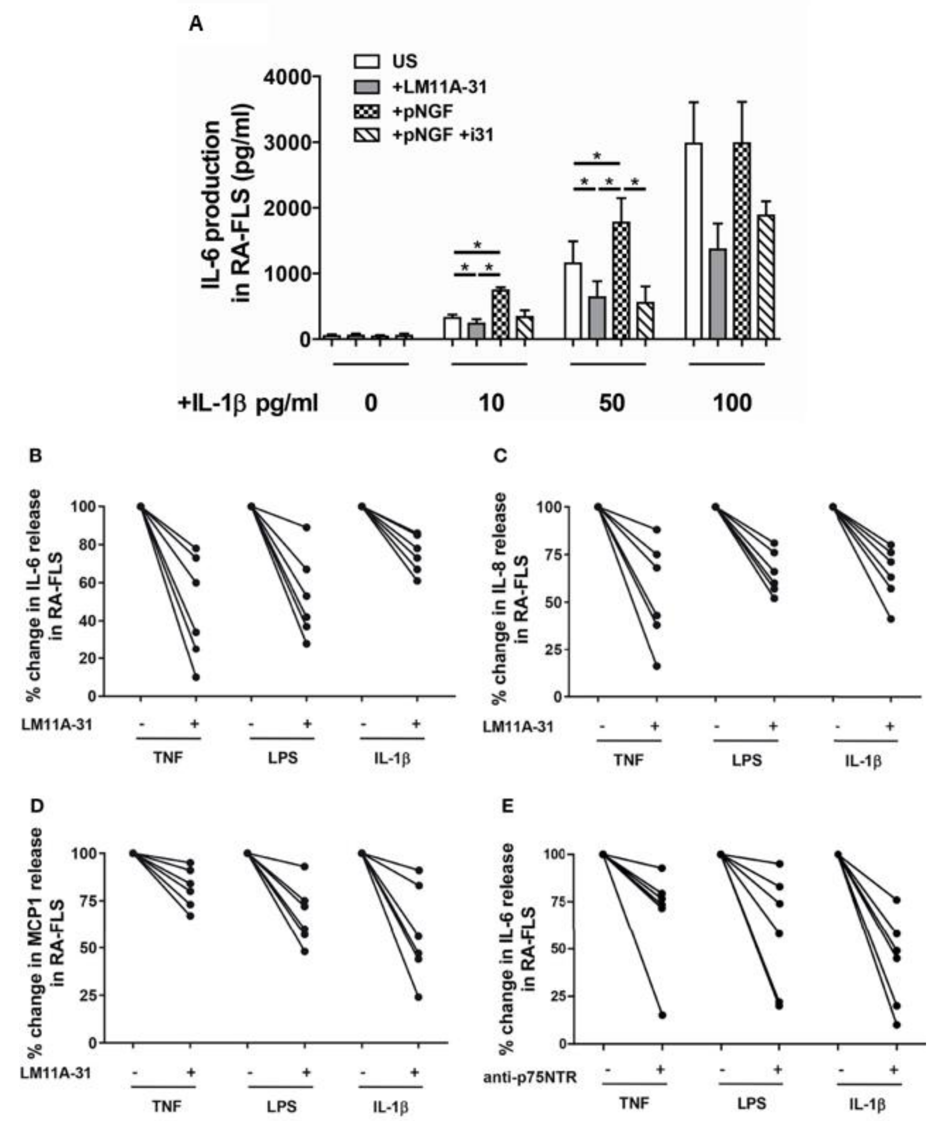

Figure 16. proNGF increases the expression of inflammatory cytokines in synovial fibroblasts from RA patients. A) The addition of exogenous proNGF (200ng/ml) to RA-FLS, cultured in a chemically defined medium, enhances the production of IL-6 in RA-FLS activated with sub-optimal doses of IL-1β (IL-1β 10-50-100 pg/ml). The increase of IL-6 induced by exogenous proNGF (IL-1 β +pNGF) is blocked by p75NTR inhibition with 10nM LM11A-31 (IL-1β +i31). IL-1β-activated RA-FLS show a similar IL-6 reduction when p75NTR was inhibited (IL-1β +i31). The experiments (n = 4) were performed in serum free medium. Data were analyzed by paired t-test (*$p < 0.05$). B–D) Inhibition of proNGF binding to p75NTR with LM11A-31 (10nM) decreases inflammatory mediators: IL-6 B), IL-8 C) and MCP1 D) production was significantly reduced in RA-FLS cells activated using 100 ng/ml TNF-α, 100 ng/ml LPS or 1 ng/ml IL-1β. E) To obtain a confirm of the effects of p75NTR blocking, p75NTR was neutralized using a specific anti-p75NTR antibody (2,5 µg/ml) and the release of IL-6 was measured in RA-FLS activated with 100 ng/ml TNF-α, 100 ng/ml LPS or 1ng/ml IL-1β. In this set of experiments (n = 6) cells were cultured in 10% FBS DMEM for 18 hours. The inhibitory effect of LM11A-31 on cytokine release are expressed as percentage decrease (% decrease) from activated cells [173]

Summarizing all these *in vitro* data, RA-FLS activated by inflammatory cytokines overexpress p75NTR and, at the same time, release increased amounts of proNGF which, by binding to p75NTR, further up-regulates inflammatory cytokine production. Neutralization of p75NTR breaks this pro-inflammatory loop and significantly reduces inflammatory cytokine production **(Figure 17).**

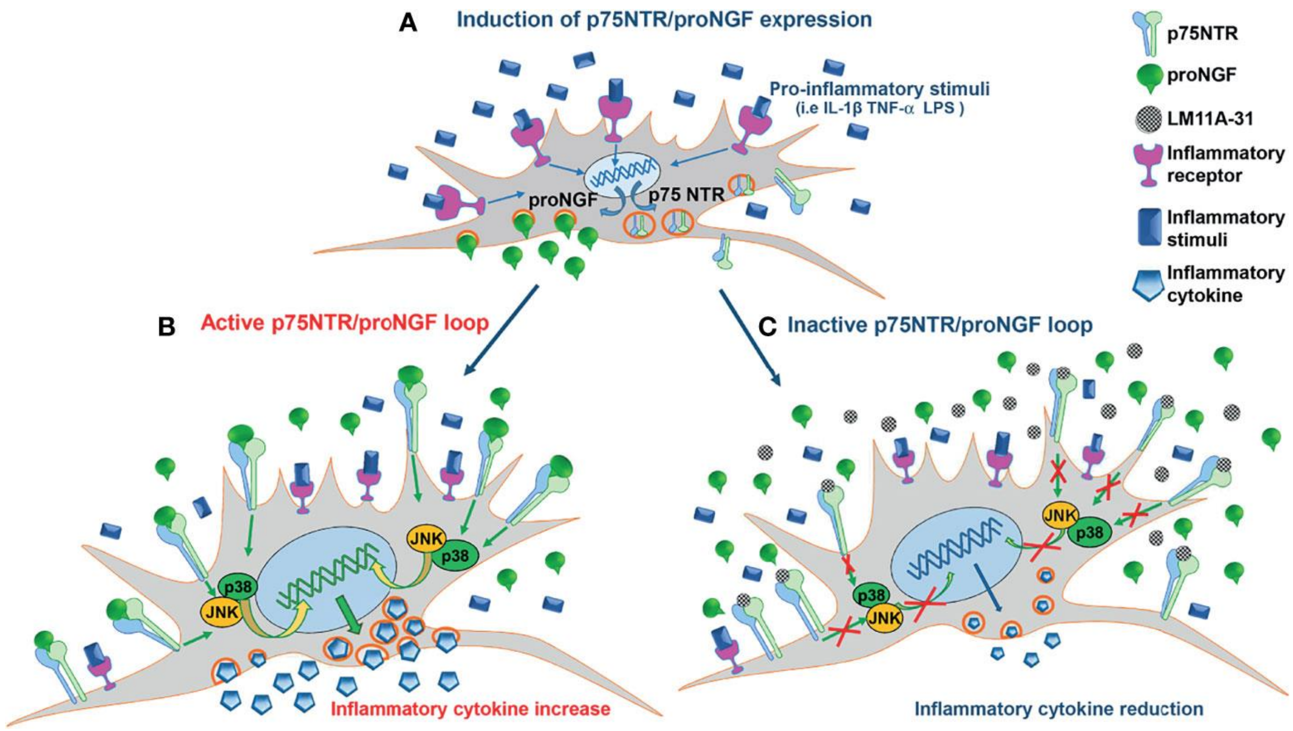

Figure 17. The pro-inflammatory p75NTR/proNGF loop in RA-FLS. (A) Inflammatory stimuli activate an autocrine loop involving proNGF and p75NTR. Inflammatory stimuli strongly induce the contemporary expression of p75NTR, the specific proNGF receptor, and of its ligand, proNGF, in RA-FLS. The increased p75NTR expression results in a higher binding capacity of RA-FLS for proNGF, whose endogenous production is strongly induced by inflammatory stimuli. (B) The active p75NTR/proNGF axis enhances inflammatory cytokines production. The high amounts of endogenously-produced proNGF, released by activated RA-FLS, interact with the p75NTR receptors highly expressed on RA-FLS membrane. The activation of p75NTR intracellular pathways (i.e. p38 and JNK) results in an amplified production of inflammatory cytokines (i.e. IL-6). (C) Inhibition of the p75NTR/proNGF loop decreases inflammatory cytokines production. The blocking of p75NTR using LM11A-31, a small molecule that specifically blocks the binding site of p75NTR for proNGF, results in a net reduction of inflammatory cytokine production (i.e. IL-6). The endogenous proNGF released by active RA-FLS cannot bind to p75NTR and, consequently, does not activate p75NTR intracellular pathways that induce inflammatory cytokine production.[173]

7.4 proNGF present in synovial fluid induces inflammatory cytokine production from RA-FLS

To better characterize the role of an active p75NTR/proNGF axis we used a more physiological culture system[176], to recreate, at least partially, the pathological conditions of an inflamed synovium by culturing RA-FLS in media supplemented with 30% synovial fluid (SF) obtained from arthritis patients. Rather than using a chemically defined medium and only one stimulus, such as a single recombinant cytokine, this *ex vivo* model provides a means to activate FLS with the mixture of pro-inflammatory and anti-inflammatory mediators that are present in the synovial fluids (SF) of inflamed joints. In addition to that, SF of JIA and RA patients are characterized by high concentrations of proNGF and markedly lower concentrations of mature NGF [161] **(Figure 18A)**. Using SF to cultivate RA-FLS swe observed a significantly increase in production of IL-6. The induction of IL-6 synthesis is mediated almost completely by the proNGF present in SF **(Figure 18B).** The addition of the p75NTR inhibitor LM11A-31, by blocking the binding of the proNGF present in SF to p75NTR (highly expressed in RA-FLS), led to an 80% reduction of IL-6 release induced by synovial fluid stimulation **(Figure 18C)**. Since it is well known that IL-1β is a potent activator of RA-FLS [157], in these experiments we neutralized the IL-1β present in the inflamed synovial fluid by using canakinumab. We found that neutralization of p75NTR using LM11A-31 was as effective as IL-1β inhibition with canakinumab **(Figure 18D)**. Although these data are limited to an *ex vivo* culture system, they nonetheless suggest that inhibition of p75NTR is, at least, as effective as the inhibition of a potent activator of pro-inflammatory response in RA-FLS as IL-1β.The observed decrease in cytokine production, induced by LM11A-31 blocking of p75NTR activity, is not due to any toxic effect of the p75 inhibitor or decreased survival of RA-FLS: neither 30% v/v SF nor different LM11A-31 concentrations (the highest dose was 10-fold that used in the neutralization experiments) induced an increase in the percentage of apoptotic cells in RA-FLS **(Figure 18E)** [173].

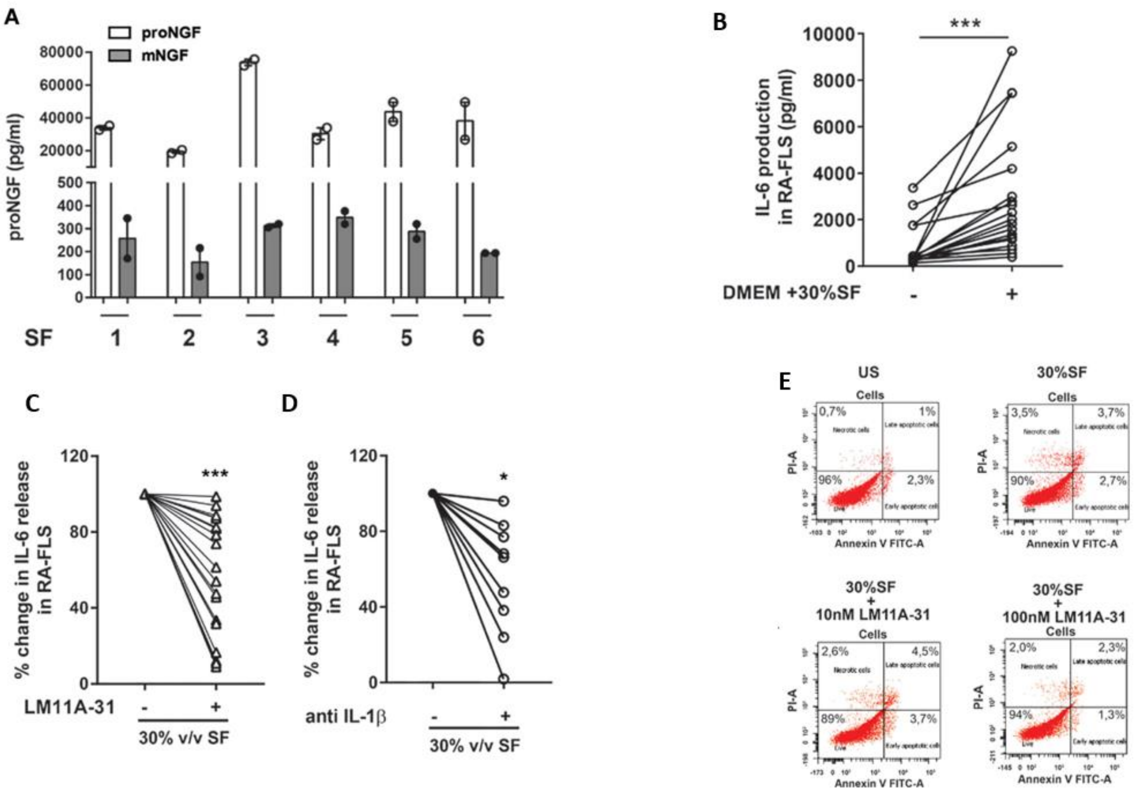

Figure 18. proNGF present in synovial fluids increases the expression of inflammatory mediators in synovial fibroblasts from RA patients. A) proNGF is by far the most abundant NGF form (100 to 200-fold that of mature NGF) detected in synovial fluids (SF) obtained from six active

patients (1-6). B) To recreate *ex vivo* inflamed synovia condition, 30% v/v synovial fluid was added to RA-FLS (n = 19) cultured in 10% FBS DMEM. Data were analyzed by paired t-test (***p < 0.001). C) RA-FLS in 30% v/v synovial fluid were treated with 10 nM of LM11A-31 with LM11A-31 and IL-6 production was measured after 18 hours. The data represent the percentage of inhibition obtained in 19 independent experiments performed using seven different RA-FLS and synovial fluids obtained from different patients (n = 19). Data were analyzed by one sample t-test (***p < 0.001). D) 30% v/v synovial fluid was added to RA-FLS cultured in 10% FBS DMEM with or without the addition of anti-IL1b (5µg/ml) (n = 9). IL-6 release was measured after 18 hours of incubation. Data were analyzed by one sample t-test (*p < 0.05). E) The apoptosis rate of RA-FLS treated with 30% v/v of synovial fluids with or without the addition of LM11A-31 at two different doses (10nM used for all our experiment and a ten-fold higher dose 100 nM) was analyzed by Annexin V/PI staining. No modification in the percentage of apoptotic cells was observed following the addition of synovial fluid or after p75NTR inhibition with LM11A-3 with both the doses used. Representative scatterplots of three independent experiments are shown[173].

To gain insights into the intracellular mechanisms regulated by p75NTR activation in this culture system and to assess whether p75NTR activity modulates the pro-inflammatory signaling response, we focused on two key mediators of pro-inflammatory cytokine production in RA synovium the p38 and JNK proteins, members of the MAPK pathways [177]. RA-FLS cultured in 30%SF showed an increase in p38 and JNK phosphorylation. This effect is mediated by the proNGF present in inflamed SF, since p75NTR neutralization significantly reduced p38 and JNK phosphorylation, demonstrating involvement of MAPK pathway in p75NTR signal transduction in RA-FLS. This observation is consistent with the known role of these MAPKs in mediating pro-inflammatory responses [178]. **(Figure 19)**.

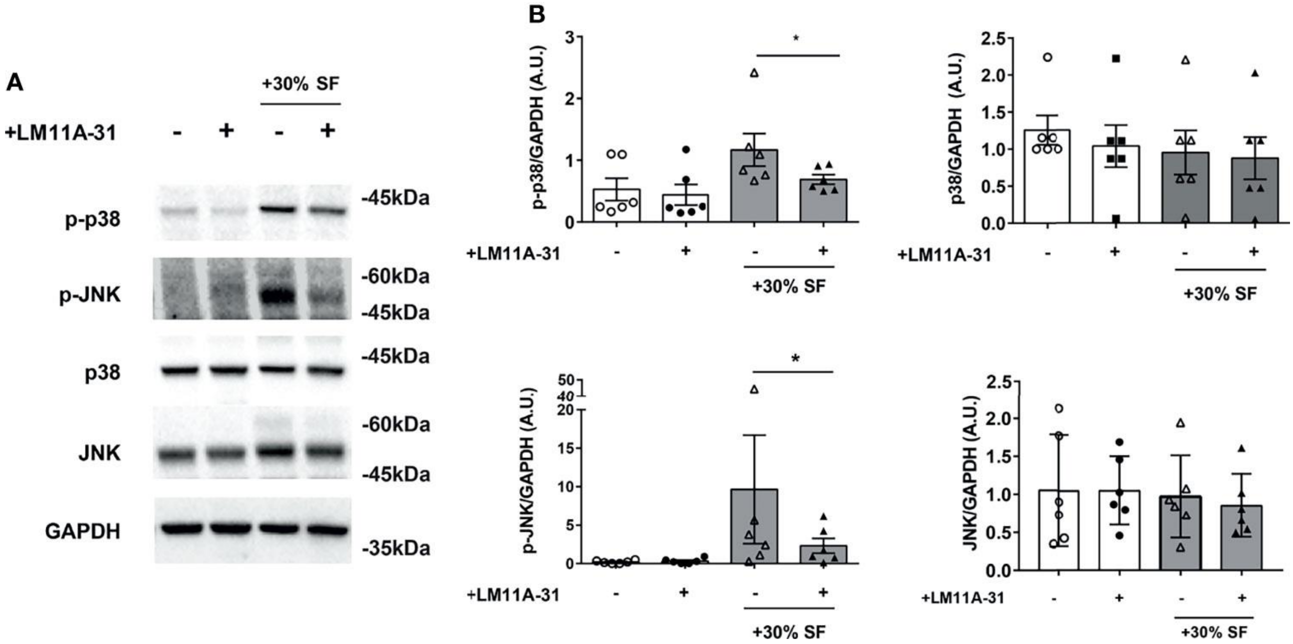

Figure 19. Activation of p38 and JNK is reduced by LM11A-31 inhibition of p75NTR in RA FLS A) RA-FLS were starved for 3 hours and incubated with 10% FBS-DMEM supplemented with 30% v/v synovial fluid and with or without LM11A-31 (10nM) for 18 hours. A reduction in the phosphorylation of p38 and JNK was observed after LM11A-31 inhibition of p75NTR activity. GAPDH was used as loading control. B) Densitometric analysis was normalized to the corresponding band intensity of GAPDH. Data were analyzed by paired t-test (*p < 0.05) and represent mean ± SEM of 6 independent experiments. Results are expressed as arbitrary units (A.U.) [173].

8. *In vivo:* p75^{NTR} expression in Antigen-Induced Arthritis (AIA) model

To assess whether p75NTR activation plays a role in the onset of arthritis and in joint inflammation we used a well-known animal model of arthritis, the antigen-induced arthritis mouse model (AIA) **(Figure 20A)**. The analysis of inflamed AIA knee joints showed a strong p75NTR IHC staining **(Figure 20B)** that was highest in infiltrated immune cells and along synovial membrane **(Figure 20B**). As AIA model is characterized by a local inflammation which is associated to an increase of spleen dimension, we analyzed also the expression of p75NTR in this lymphoid organ. We found that the basal expression of p75NTR in control mice is distributed in the red pulp and around the follicles but in AIA mice we observed an increase of positivity in all the areas and a strong positivity appeared in the follicles **(Figure 20C**). To evaluate the levels of p75NTR expression in the spleen populations of AIA mice, we performed a multicolor Flow cytometry analysis on splenic mononuclear cells isolated from individual mice.

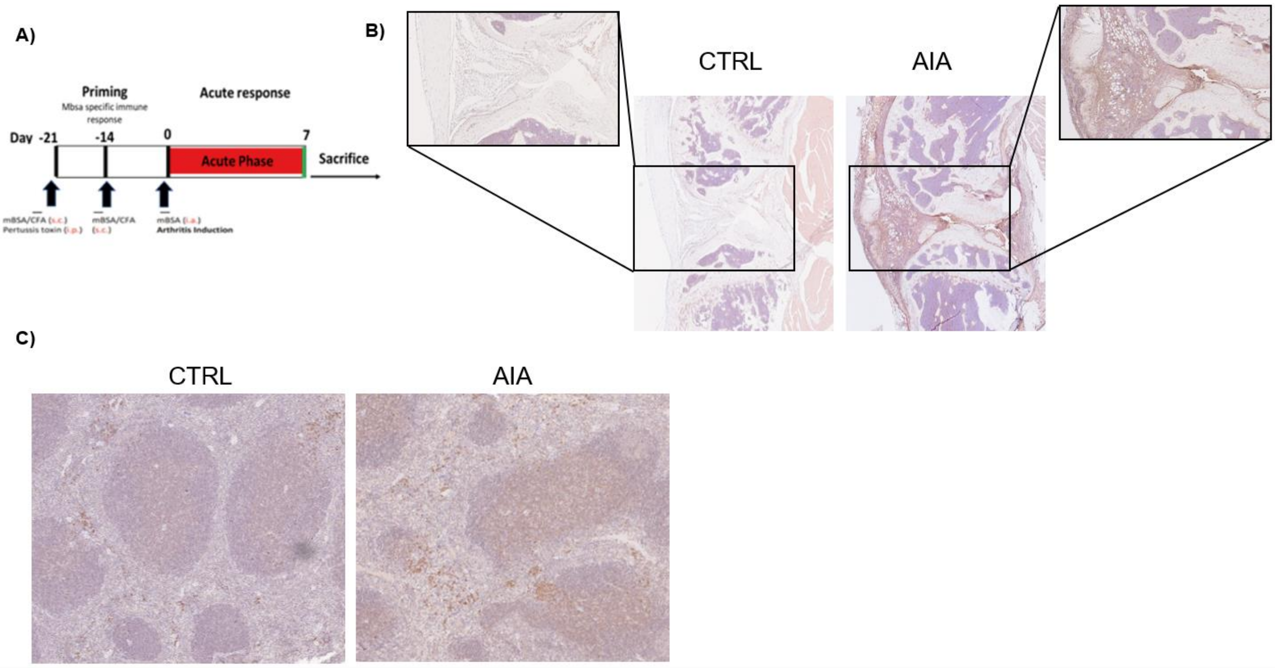

Figure 20. p75NTR expression in Antigen-induced arthritis model of inflammatory arthritis. A) Timeline of the AIA model showing the progression of the disease and treatments. Control group received an injection of PBS into the left knee joint (CTRL), while arthritis is induced by intra-articular injection of methylated BSA in the right knee joint (AIA), in order to induce the acute phase of proinflammatory response t=0. AIA-treated mice received a solution of 50 mg/kg of LM11A-31 for 7 days by oral gavage. All mice were sacrificed at day 7. p75NTR IHC staining of CTRL and AIA mice synovia B) and spleen C).

To better characterize the expression of p75NTR in the spleen populations of AIA mice, we analyzed p75NTR expression in isolated splenic mononuclear cells from individual mice. Mean fluorescence intensity (MFI) as well as the frequency of p75NTR positive cells were analyzed by multiparametric flow cytometry analysis. As shown in **Figure 21**, the induction of AIA modified the percentage of splenocyte population with a decrease in the total percent of B and T lymphocytes and a concomitant increase of innate immune cells (monocytes and DCs) in the spleen of AIA mice.

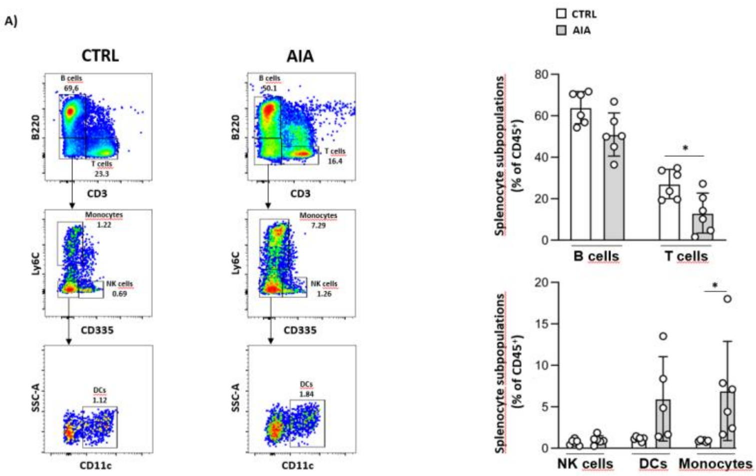

Figure 21. Characterization of splenocyte subpopulations in spleen of murine AIA model. A) The distribution of splenocyte subpopulations was analyzed on spleen mononuclear cell populations by multiparameter flow cytometry. Isolated splenocytes from CTRL and AIA mice were stained with anti-CD45/APC, anti-B220/BV510, anti-CD3/BB700, anti-Ly6c/BV421, anti-CD335/BUV395 and anti-CD11c/BUV805. A representative experiment is shown (A, left). Data show mean ± SEM.

Low basal expression of p75NTR characterized all CD45+ splenic cell population in control mice but the mean fluorescence intensity (MFI) was significantly enhanced in $CD45^+$ cells of AIA mice. Within $CD45^+$ cell population, innate (NK cells, DCs, monocytes) and adaptive immunity cells (T cells, B cells) all show enhanced levels of p75NTR expression in AIA mice. Dendritic cells (DCs) and monocytes show the highest increased of p75NTR expression compare to the other populations analysed **(Figure 22 A-C)**. These innate immune cells also showed the highest increase in the percentage of p75NTR positive cells. Taken together, these results indicate that inflammation induced in AIA mice promotes p75NTR expression on splenic immune subpopulations.

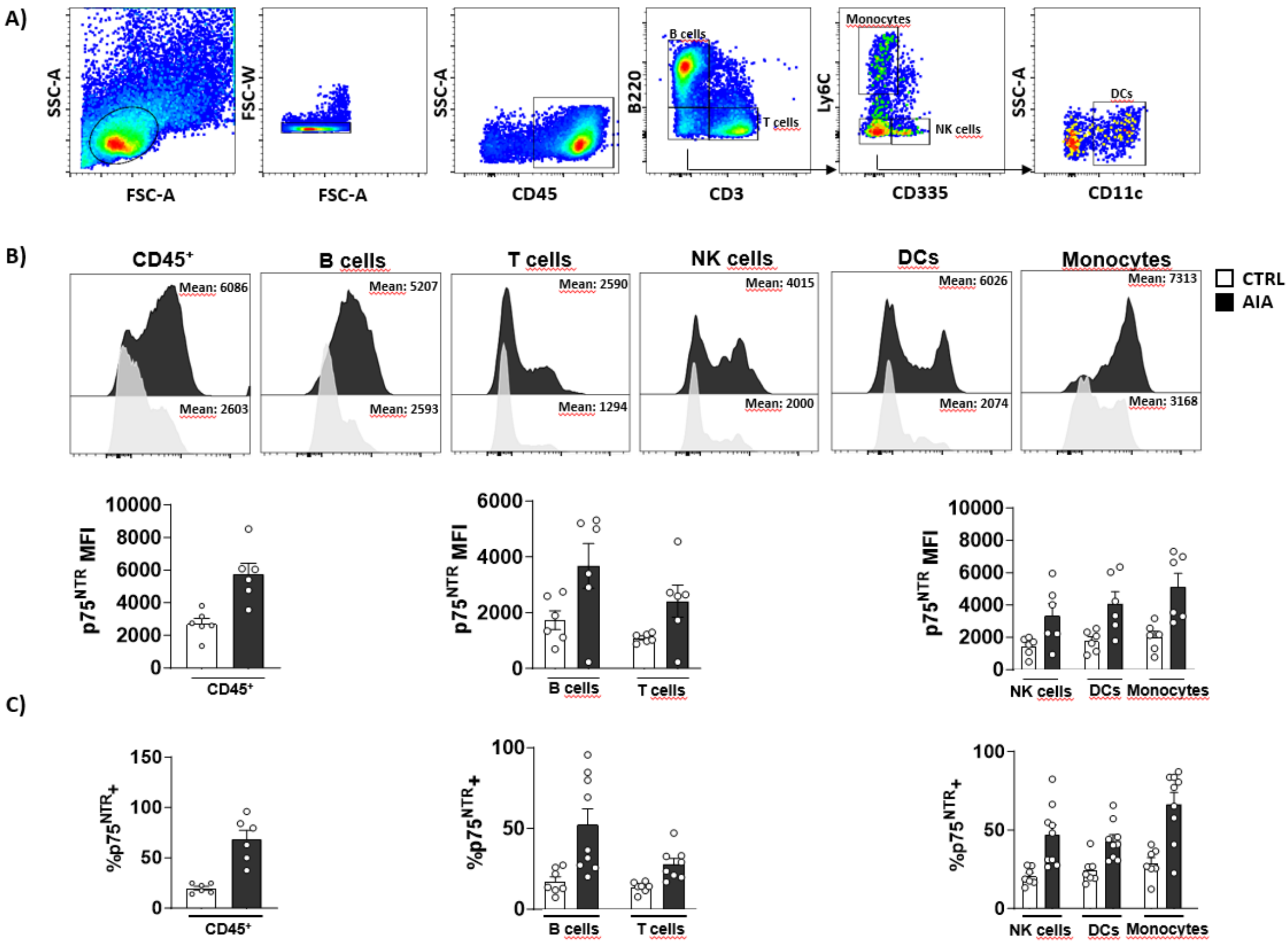

Figure 22. Characterization of p75NTR expression on splenocyte populations in murine AIA model. The expression of p75NTR was analyzed on spleen mononuclear cell poulations by multiparameter flow cytometry. Isolated splenocytes from CTRL and AIA mice were stained with anti-CD45/APC, anti-B220/BV510, anti-CD3/BB700, anti-Ly6c/BV421, anti-CD335/BUV395, anti-CD11c/BUV805 and anti-CD271/PE mAbs. (A) Gating strategy is shown. Results were expressed both as mean fluorescence intensity (MFI) (B) and percentage of positive cells (C). (B) A representative experiment is shown. Data show mean ± SD.

8.1 AIA inhibition by LM11A-31 treatment.

In order to evaluate the effect of p75NTR inhibition on arthritis development, AIA mice were divided in two groups and for seven days they received either a solution of 50mg/kg of LM11A-31 or PBS by oral gavage. As shown in **Figure 23A**, AIA mice treated daily with LM11A-31 oral gavage (AIA-treated) revealed a significant amelioration in joint swelling at day 1 (*$p<0,05$), day 2 (**$p<0,01$) and day 3 (*$p<0,05$). AIA-treated mice were characterized by a decreased joint inflammation score, associated to a reduced inflammatory cell infiltration and invasion of pannus tissue **(Figure 23B)**. Accordingly, the increase of proinflammatory cytokines, such as IL-6, IL-1β that drive inflammation in AIA mice, was abolished in the knee by LM11A-31 treatment **(Figure 23C)**. Interestingly, TNF-α did not show significant change in its expression neither in AIA or in AIA-treated mice confirming that in AIA model TNF is not the main driver of inflammatory responses in comparison to IL-6 and IL-1β [179]**(Figure 23C)**.

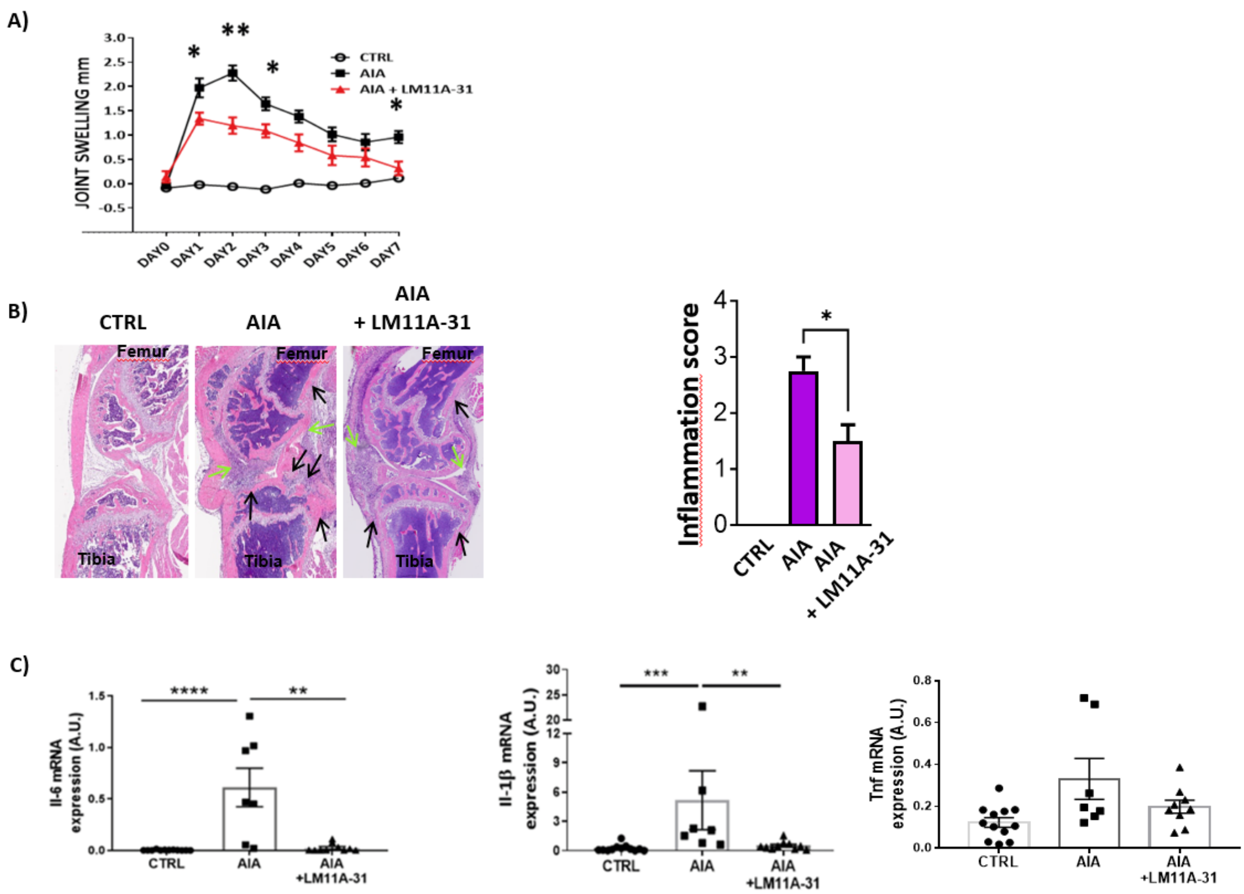

Figure 23. Inhibition of p75NTR in inflamed synovia by LM11A-31 A) For a clinical evaluation of arthritis, joint swelling was quantified (in mm) using a micrometer. The data represent the difference between control left knee and arthritic right knee (n=14). AIA mice treated daily with LM11A-31 oral gavage show a significant amelioration in joint swelling at day 1 (*p<0,05), day 2 (**p<0,01) and day three (p<*0,001). B left) Representative images of Hematoxylin and Eosin (H&E) staining of control and AIA mice. Blue nuclei of cells indicate synovial joint inflammation which is characterized by inflammatory cell infiltrates and invasion of pannus tissue (Green arrows). H&E staining also allows morphological discrimination of intact bone surface and bone erosions (black arrows). Original magnification 1.5 X. B right) Scoring of synovial joint inflammation in untreated and inhibitor-treated AIA mice. The degree of synovial inflammation was scored ranging from 0 (healthy) to 3 (severe). C) To evaluate the local effect of LM11A-31 treatment, IL-1β, IL-6 and TNF-α mRNA expression was analyzed in the synoviae after 7 days of treatment.

Toluidine blue staining of arthritic joints showed reduced proteoglycan content in AIA mice as revealed by destaining of the superficial cartilage that is consistent with the cartilage damage in AIA mice; interestingly, LM11A-31 treatment reduced the extent of proteoglycan loss-associated destaining and significantly reduce the percentage of eroded surface on total bone surface in LM11A-31-treated AIA when compared to AIA mice **(Figure 24A)**. Consistently with this finding, the histochemical analysis for the osteoclast-specific marker TRAcP revealed lower levels of synovial TRAcP-positive cells in both tibial proximal epiphysis and femoral distal epiphysis of LM11A-31 treated animals **(Figure 24B left)**. Moreover the histomorphometric analysis showed a trend of reduction in osteoclast number (Oc.N) and surface (Oc.S) of cartilage covered by osteoclasts in LM11A-31-treated AIA mice compared to vehicle treated AIA mice **(Figure 24B right).**

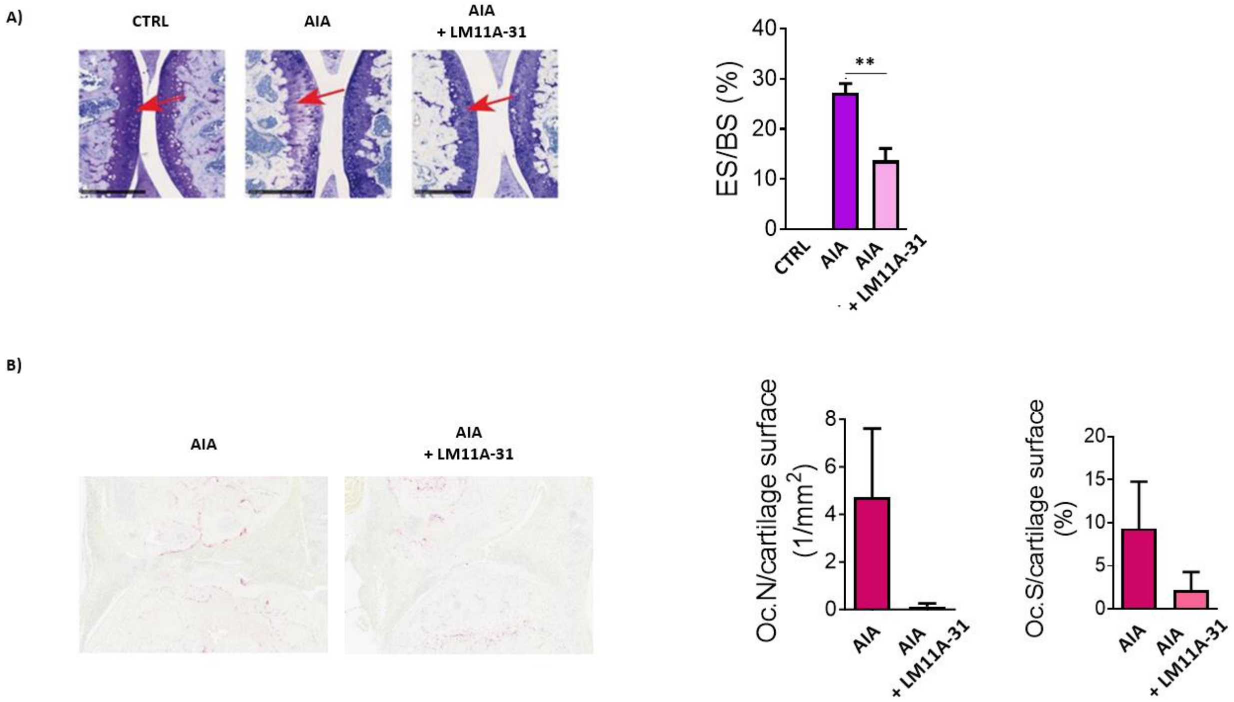

Figure 24. Evaluation of cartilage erosion by osteoclasts in arthritic joints. A left) Representative images of toluidine blue staining and A right) quantification of arthritic joints revealing the reduction of the extent of proteoglycan loss-associated destaining in synovia samples treated with LM11A-31. B left) Representative images of TRAcP staining revealing osteoclasts on epiphyseal cartilage region and (B, right) quantification of osteoclast number *per* area counted on epiphyseal cartilage surface in femurs of untreated and inhibitor-treated AIA mice. Results are expressed as mean ± SD. *$p<0.05$. A minimum of four animal was analyzed for each group.

8.2 Effect of LM11A-31 on spleen organization

In agreement with what has been previously described in arthritis mouse models [180], AIA mice are characterized by an enlargement of the spleen volume (**Figure 25A)**, that was significantly reduced in AIA-treated mice. To verify if the observed changes in spleen volume were due to morphological rearrangement, we analyzed the tissue organization in ematossilin-eosin-stained spleens and by immunostaining of spleen sections with marginal zone macrophage (MZM) and B cells markers to identify follicle regions. Our results indicated that the perimeter and area of the follicles showed no significant differences in AIA and AIA-treated groups **(Figure 26A)**, suggesting that splenomegaly observed in AIA mice was mainly due to an increase of the red pulp area **(Figure 25B).**

In agreement with the observed splenomegaly and consistently with the augmented volume of the red pulp, the absolute number of cells was greater in the AIA mice (data not shown) with changes in splenic subpopulations. The induction of AIA modified the relative percentage of splenocyte populations with a decrease in B and T lymphocytes and an increae of percentage of monocytes **(Figure 26B-Figure 25C)**. The treatment with LM11A-31 decreases the number of inflammatory innate cells in the spleen, bringing them back to control values **(Figure 25C)**.

Since integrin CD11b (α-M integrin) plays important role during inflammation and it is considered as a marker of cell activation typically present on all myeloid cells [181], we analyzed its expression on splenocyte subpopulations. AIA splenocyte subpopulations showed a significant increase of CD11b compared to CTRL mice that is consistent with the activate state of the cells due to the inflammatory response in AIA mice. The treatment with p75NTR inhibitor, reduced instead significantly the expression of CD11b in the AIA splenocyte subpopulations, especially in DCs and monocytes **(Figure 25D)**. The reduction of the inflammatory state of AIA-treated mice is also indicated by the changes in the ratio between Th17 and Treg cells. In AIA mice there is an increase in the percentage of Th17 lymphocytes and a reduction of Treg while in AIA-treated mice the Th17/Treg ratio is restored to control levels **(Figure 25E)**. Another indication of the inhibited inflammatory axis and reduced cell activation in AIA-treated mice is the significant decrease in the plasma concentration of total IgG and mBSA-specific IgG (**Figure 25F)**.

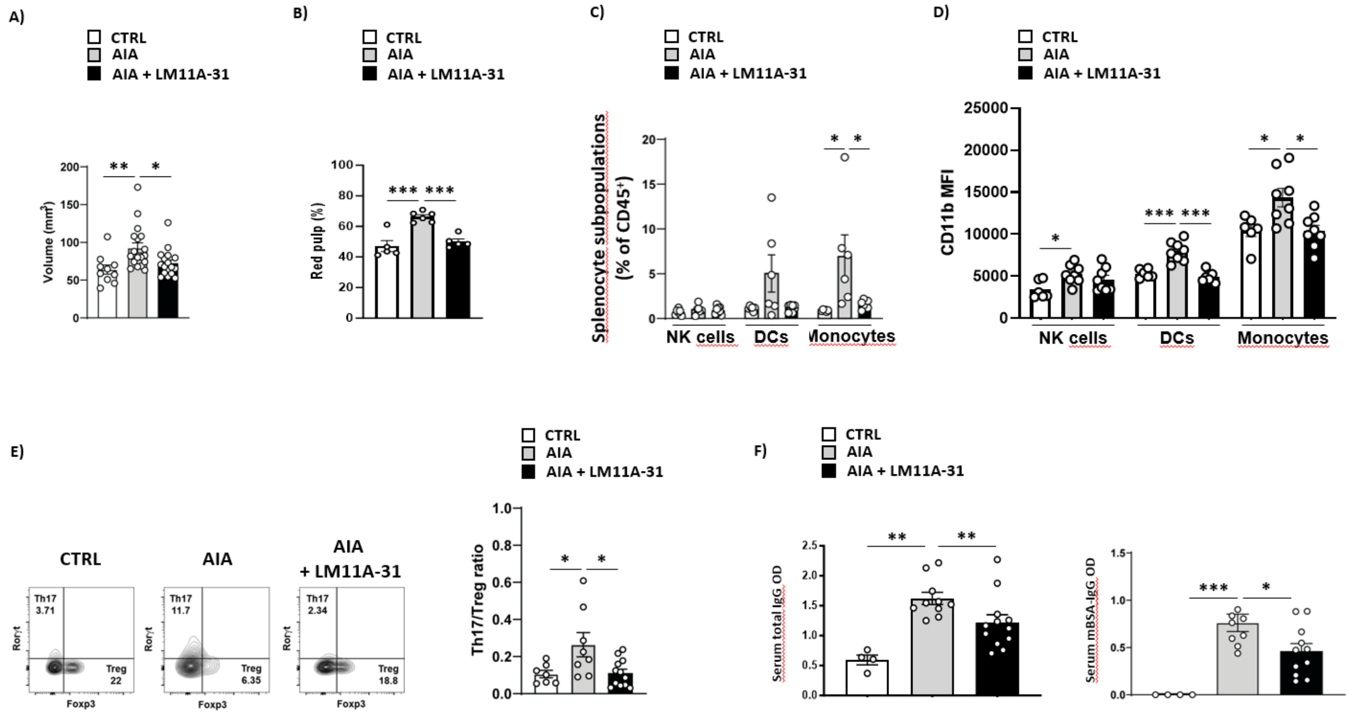

Figure 25. Inhibition of p75NTR in spleen by LM11A-31 A) Spleen volume of CTRL, AIA treated or not with LM11A-31 was measured. (B) Red pulp surface extension of spleen was calculated as percentage of total splenic surface B). C) Isolated splenocytes from WT mice and AIA model treated or not with p75NTR inhibitor LM11A-31 were subjected to multiparameter flow cytometry analysis and stained with anti-CD45/APC, anti-B220/BV510, anti-CD3/BB700, anti-Ly6C/BV421, anti-CD335/BUV395, anti CD11b/FITC, anti-CD11c/BUV805 and anti-CD271/PE mAbs (E) or with anti-CD45/BUV395, anti-CD3/BV786, anti-CD4/Pecy7, anti-CD271/PE, anti-Foxp3/APC and anti-Ror□t/PECF594. D) CD11b MFI was calculated on splenocyte subpopulations. E) Reduction of Th17/Treg ratio in AIA treated with LM11A-31 rescued to CTRL levels. F) Serum levels of total and mBSA-specific IgG were evaluated by ELISA assays. Data show mean ± SEM.

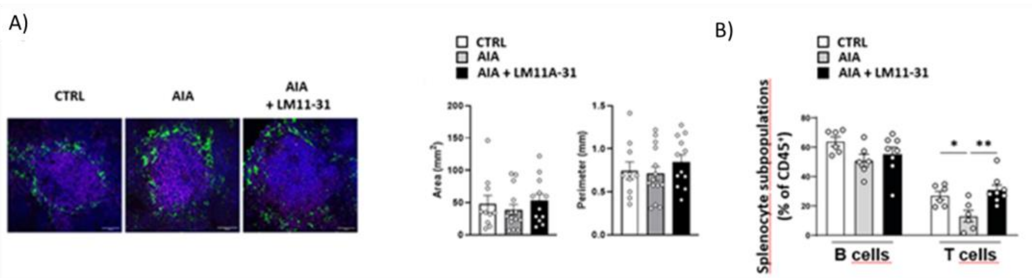

Figure 26. Characterization of splenocyte subpopulations in spleen of murine AIA model and analysis of inhibition of p75NTR in spleen by LM11A-31. A, Morphological analysis of follicles in CTRL, AIA treated or not with LM11A-31 was evaluated by an immunostaining of spleen sections with anti-MARCO and anti-B220 mAbs. (A, right) Area and perimeter of follicles were calculated (n=10-14 follicles measure in 4 different animals for each experimental group). B) The effect of LM11A-31 on splenocyte supopulation distribution was evaluated spleen mononuclear cell poulations by multiparameter flow cytometry. Isolated splenocytes from CTRL and AIA mice were stained with anti-CD45/APC, anti-B220/BV510, anti-CD3/BB700, anti-Ly6c/BV421, anti-CD335/BUV395 and anti-CD11c/BUV805.

9. p75NTR as a possibile novel diagnostic biomarkers in arthitis patients

As mentioned before, enhanced expression of p75NTR and the concomitant increase in p75NTR-ECD are considered as potential biomarkers in several diseases; in fact, there is evidence for increased p75NTR-ECD levels in neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis, age-related dementia, schizophrenia, and diabetic neuropathy. The accumulation of p75ECD into biological fluids such as blood, cerebrospinal fluid (CSF), and urin has been considered as an indication of the greatly enhanced expression of p75NTR in neurodegenerative diseases and its use as a biomarker for diagnosis and/or prognosis has been prospected for these disorders.

9.1 Activated synoviocytes release p75ECD

To evaluater whether p75ECD is present in the inflamed synovia we analyzed the presence of p75ECD in synovial fluids of JIA patients and, as shown in the western blot, we found that p75ECD accumulates in the synovial fluids of patients with an active disease **(Figure 27)**. This finding was confirmed *in vitro* using RA synoviocytes that were stimulated with IL-1β. In RA FLS there is proteolytic cleavage of p75NTR by α-secretase that releases the ECD in the conditioned media. The remaining membrane-bound C-terminal fragment *(p75-CTF)* is further processed by γ-secretase resulting in the release of p75 intracellular domain in the cytoplasm. Stimulation of RA-FLS with Il-1β enhances p75NTR expression and increases p75ECD accumulation in the conditioned media. Using an inhibitor of α-secretase as TAPI2, the accumulation in the conditioned media of p75ECD was strongly reduced in untreated and IL-1β stimulated RA-FLS. On the contrary there was no decrese in the release of p75ECD when the γ-secretase inhibitor DAPT was used **(Figure 27)**.

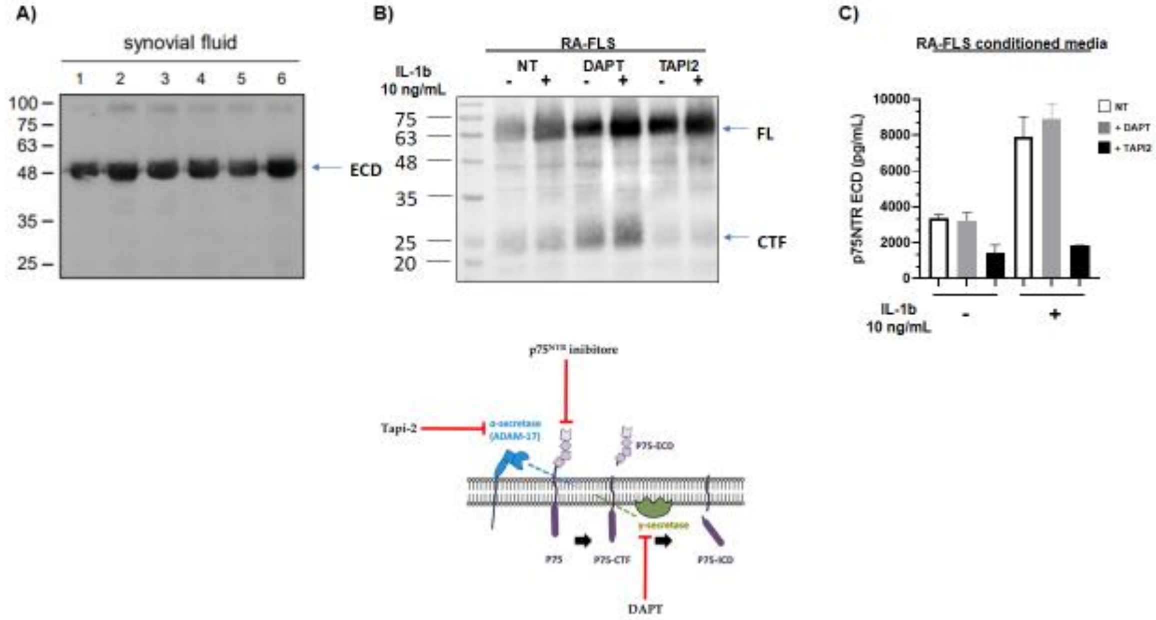

Figure 27 Proteolytic cleavage of p75NTR in RA fibroblats-like synoviocytes.A) Western Blot analysis of synovial fluids from active JIA patients revealed in the inflamed synoviae the presence of the cleaved p75ECD fragment. B) p75NTR is cleaved in RA FLS as indicated by the appearance of membrane-bound C-terminal fragment (CTF). The stimulation of RA-FLS with 10ng/ml IL-1β enhance full lenght p75NTR receptor (FL) on the plasmatic membrane and CTF formation. Blocking the α-secretase with TAPI 2 abolishes the CTF appearance that instead was not modified by γ-secretase inhibition. C) The analysis of the conditioned media confirmed the shedding of p75-ECD

9.2 Differential levels of p75NTR ectodomain (ECD) in blood and sinovial fluids of JIA patients

To assess whether p75NTR-ECD could be used as a biomarker of active inflammatory response we analyzed the levels of ECD release in plasma and synovial fluids at onset in JIA patients naive to treatment or in JIA patients under treatment and in remission state. The analysis of p75NTR-ECD concentrations showed that in JIA patient there is a significant increase in the circulating p75NTR ECD compared to healthy donors levels. Moreover, there is an accumulation of p75NTR ECD in the synovial fluids of inflamed synoviae. These concentrations are higher than the ones found in the blood of JIA patients. This is even more evident in the analysis of p75NTR ECD levels in the blood and SF of matched samples from the same patient **(Figure 28)**

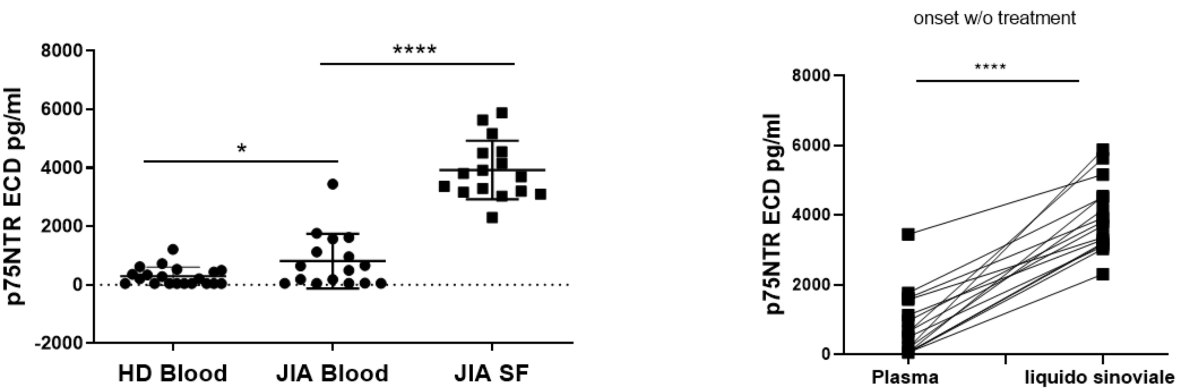

Figure 28. p75NTR-ECD in JIA patients. A) p75NTR-ECD is far more released in JIA SF compared to JIA blood and blood from healthy donors. B) Paired-graph of n=17 of p75NTR-ECD levels measured in blood and synovial fluids of JIA patients. Data were analyzed using unpaired t-test (*p < 0.05, ***p<0.001)

In order to better understand if p75NTR-ECD may be linked to progression of the disease we analyzed p75NTR ECD in the blood of patients with different JIA classification. We demonstrated that, if compared to plasma levels in CTRLs, ECD is by far more concentrated in JIA plasma of patients at the onset and naive to treatment, while there is a significant down modulation of the ECD production during the remission on therapy in JIA patients, thus indicating p75NTR could associated to an active state of arthritis **(Figure 29)**.

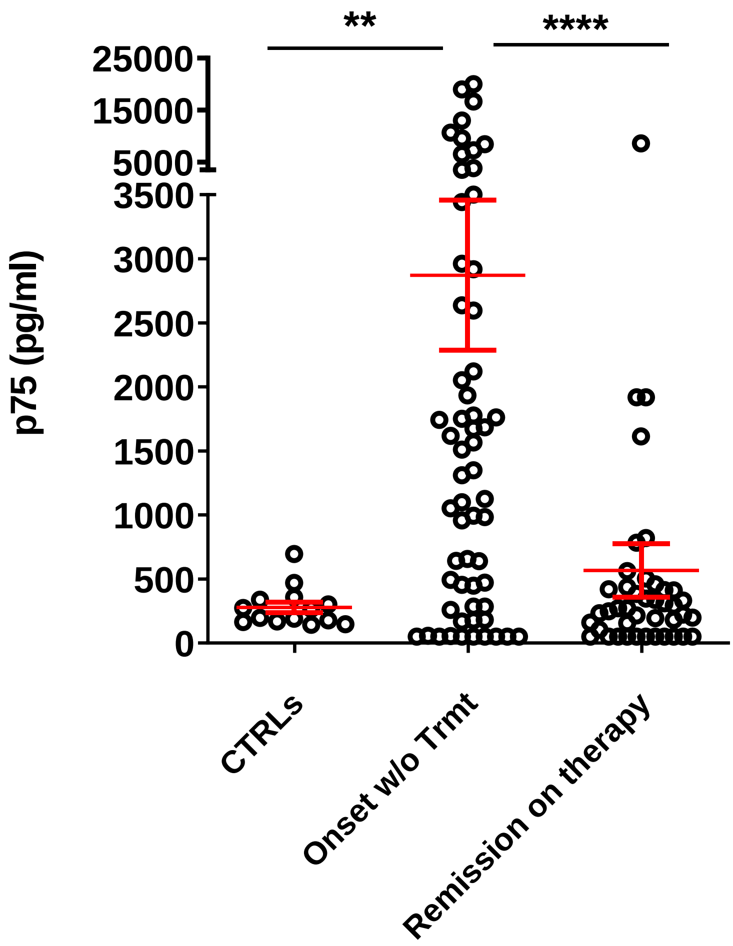

Figure 29. p75NTR-ECD distribution in different JIA classification: ELISA test for p75NTR-ECD revelad a more concentration of ECD in plasma of JIA patients at onset w/o treatment (n=61) if compared to JIA plasma collected in remission oh therapy state (n=38) and to CTRL plasma group (n=15). Data were analyzed using unpaired t-test (**p < 0.01, ***p<0.001)

Considering the cytokines that play a key role in JIA pathogenesis, for a full assessment of the inflammatory process in the JIA patients, we analyzed the circulating levels of pro-inflammatory cytokines such as IL-6 and TNF-α [182] during pathology development and correlated them with the p75NTR-ECD levels, measured in the blood of JIA patients. We found that in JIA patients at onset and without any kind of treatments, p75NTR-ECD levels are directly proportional to the amount of circulating pro-inflammatory cytokines as IL-6 and TNF-α. **(Figure 30)**

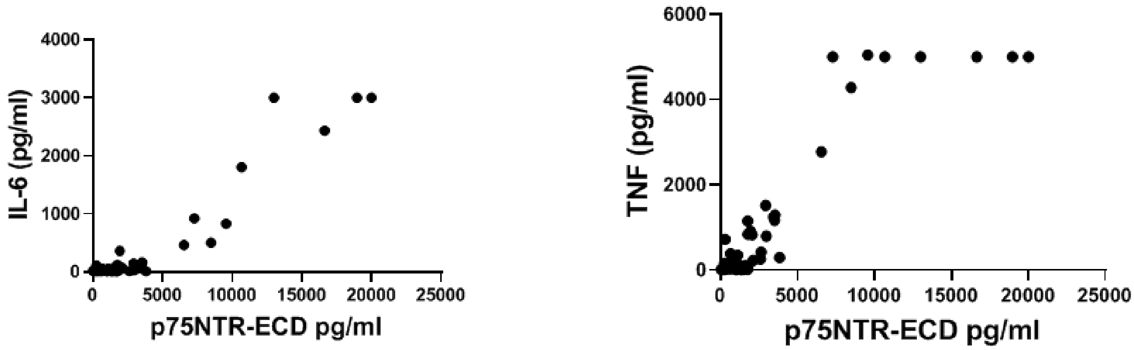

Figure 30. p75NTR-ECD in correlation with IL-6 and TNF in plasma of JIA patients at onset w/o treatments. Ectodomain shedding of p75NTR shows a directly correlation with IL-6 and TNF proinflammaory cytokines measured in ELISA test in plasma blood from JIA patients at onset. Spearman analysis were used showing an r value of 0.6620 and a p value (two tailed) <0.0001 for p75NTR-ECD and IL-6, and r value of 0.7972 and a p value (two tailed) <0.0001 for p75NTR-ECD and TNF.

10. Discussion and Conclusions:

Despite currently available targeted therapies, many patients with RA or JIA do not achieve remission, instead accruing functional and structural damage. It is therefore important to better understand the mechanisms and mediators that lead to disease chronicity and that are not inhibited in non-responder patients by current therapies. The identification of new mechanisms of action will help to define compounds for novel therapeutic approaches. In the course of the present study, we have identified a novel pro-inflammatory role of p75NTR and its ligand proNGF, both involved in the amplification of synovial inflammatory responses. At disease onset, in the inflamed synovium of RA patients, two distinct clusters of FLS with specific tissue localization (sub lining THY1$^+$COLA1$^+$and lining PGR4$^+$ FLS) known to drive inflammation and damage [172], show the highest expression levels of p75NTR and of its co-receptor sortilin when compared to other synovial tissue cells. Moreover, the comparative analysis at single cell level of synovial tissues from treatment-naïve and in remission RA patients showed that p75NTR expression is significantly down regulated once sustained remission is achieved, supporting the notion that activation of proNGF/p75NTR axis is involved in RA synovial inflammation. Complementing these findings, in *in vitro* studies, we confirmed that RA-FLS have a markedly higher basal expression of p75NTR and sortilin than skin fibroblasts or OA-FLS, indicating the presence in RA patients of a functional high-affinity proNGF receptor complex. Similarly to what has been described in blood and synovial fluid-derived immune cells [161], inflammatory stimuli, such as LPS and the classical pro-inflammatory cytokines as IL-1β and TNF-α, enhance the expression of p75NTR in RA-FLS, suggesting a strict relationship between inflammation and the overexpression of p75NTR. FLS isolated from both RA and JIA patients are characterized not only by a high p75NTR but also by a low TrkA expression that may amplify the pro-inflammatory effects of proNGF since the anti-inflammatory actions of mature NGF mediated through TrkA activation is dampened. Further supporting the finding of an active proNGF7p75NTR axis in JIA and RA patients, we demonstrated that in the synovial fluids from inflamed joints of there are high levels of proNGF, but not of mature NGF. The accumulation of proNGF in the synovial fluid is due to pro-inflammatory stimuli that not only enhance p75NTR expression but also induce proNGF production in RA FLS as wells as in synovial mononuclear cells. As RA-FLS have a pro-inflammatory and aggressive phenotype [183] and a number of epigenetic changes have been suggested to account for these abnormalities [184], it is tempting to speculate that p75NTR overexpression and proNGF overproduction contribute to the abnormal pro-inflammatory phenotype in RA-FLS and, possibly, to the underlying epigenetic changes. Our results have demonstrated that hyperactivation of p75NTR pathway, is, at least in part, responsible for the known prolonged production of inflammatory cytokines and neutralization of p75NTR activity, using LM11A-31, a small non peptide molecule that selectively blocks the binding of proNGF to p75NTR and its signalling, breaks this pro-inflammatory loop. Indeed, the blocking of the binding of the endogenously-produced proNGF to p75NTR in IL-1β-activated RA-FLS resulted in a marked decrease in inflammatory cytokine production, indicating the functional interaction of p75NTR and proNGF and confirming the presence of a pro-inflammatory autocrine loop. To validate these findings using a more physiological culture system, we recreated, at least partially, the pathological conditions of an inflamed synovium by culturing RA-FLS in media supplemented with 30% synovial fluid (SF) obtained from arthritis patients. We found that the addition of SF to RA-FLS significantly increased the production of IL-6. This effect is mediated almost completely by the proNGF present in SF, as the inhibition of proNGF binding to p75NTR led to an 80% reduction in IL-6 production. As it is well known that IL-1β enhances the expression of various inflammatory factors in RA-FLS through the activation of NF-kB/ERK/STAT1 axis, we compared in this cellular model the effects of IL-1β and p75NTR neutralization on inflammatory cytokine production. We found that in RA-FLS cultured with 30% SF the inhibition of p75NTR activation by LM11A-31 reduces the release of IL-6 to a similar extent as IL-1β inhibition by canakinumab, a monoclonal antibody targeting IL-1β used for the treatment of RA. Although these data are limited to an *ex vivo* culture system, they nonetheless suggest that inhibition of p75NTR is, at least, as effective as the inhibition of a potent activator of pro-inflammatory response in RA-FLS as IL-1β. The analysis of p75NTR signal transduction in RA-FLS showed that inhibition of p75NTR by LM11A-31 effectively reduces phosphorylation of both p38 and JNK, MAPK that have a well- known role in activating pro-inflammatory responses. Our findings are in agreement with data obtained in other inflammatory disease showing the involvement of p75NTR in regulating inflammatory pathways. In murine streptozotocin-induced diabetes model, accumulation of proNGF and enhanced p75NTR expression have been found in the retina but p75NTR-KO mice are protected from diabetes-induced retinal inflammation and show decreased TNF-α expression [164]. Similarly, the treatment of with LM11A-31 significantly reduces the increase of serum TNF-α and IL-1β in streptozotocin-treated mice[185]. In a murine model of sepsis-induced neuroinflammation, LM11A-31 treatment causes a decrease in IL-1β concentrations in the hippocampus, that is associated with a reduction in JNK phosphorylation [185].

In order to provide more evidence on the role played by p75NTR in inducing an inflammatory response, we used a well-known animal model of arthtiris, the antigen-induced arthritis (AIA) mouse model. As our data in AIA mice indicate, the inflammatory state is indeed associated to the up regulation of p75NTR in the synovia: in the inflamed knee joint there is a striking enhancement of p75NTR expression in the synovial layer and in the inflammatory infiltrating cells, in agreement with what we have previously observed in single cell analysis of RA biopsies. The inflammatory response has an effect also on the expression levels of p75NTR in the spleen of AIA mice that were enhanced specifically in the red pulp area, where activated monocyes are localized, and in the expanded follicles. A more quantitative analysis of the splenocytes population revealed an enhancement of p75NTR positivity in the CD45+ splenocytes and in the innate and adaptative cell populations with the highest p75NTR expression in monocytes that play a crucial role in the inflammatory response. These *in vivo* results are in agreement with the well-known upregulation of p75NTR in several inflammatory dieases, such as Multiple Sclerosis (MS) disease, inflammatory Bowel disease (IBD), including Crohn’s disease and ulcerative colitis, in which p75NTR contributes to tissue damage and inflammation through immune cell activation and pro inflammatory cytokine release. Moreover, p75NTR is upregulated in patients with chronic asthma, where it contributes to the activation of mast cells and promotes the release of histamines and other inflammatory mediators, worsening airway hyperresponsiveness and chronic inflammation [186], strongly supporting the hypothesis that enhancement of p75NTR is strongly correlated to the inflammatory process.

To confirm whether the inflammation-induced up-regulation of p75NTR has a role in joint inflammation *in vivo*, we induced AIA and treated AIA mice with an oral administration of LM11A-31. After the first day of oral treatment, we start measuring a significant decrease in the knee swelling that was even more evident in the following days. The significantly reduced swelling was associated to a low inflammatory score in AIA-treated mice that was associated to a histological evaluation of decreased bone reabsorption and reduced cartilage damage. Together with the lack of inflammation-induced morphological changes in the knee, we found in the synoviae of AIA-treated mice a striking decrease in the expression of IL-6 and IL-1β, more down-regulated than TNF-α, confirming the key role of these two cytokines in driving the inflammation in AIA animal model. Together with the decrease of inflammatory cytokines in the synovia we observed a significant reduction in immune cell infiltration and activation of osteoclasts. These findings are consistent with previous studies on AIA induction in IL-6 mice deficient [187] or in AIA mice treated with anti-IL6 receptor that showed a reduced inflammatory score and histological signs of inflammation, defective production of the osteoclastogenic cytokine IL-17, decreased osteoclast differentiation, recruitment and activation.

Inhibition of p75NTR has then the general effect of dampening joint inflammation by simultaneously acting on synovial fibroblasts and immune infiltrating cells, all expressing p75NTR receptors, and reducing the local production of inflammatory cytokines. The reduced inflammation, due to LM11A-31 blocking of proNGF binding to p75NTR, decreases cytokine concentration in the inflammatory milieu of AIA-treated joints but also affects the size and organization of the spleen. Consistent with the hypothesis of a reduced inflammation state in AIA-treated mice, the spleen of these mice is characterized by a decreased influx of monocytes and reduction of red pulp volume [188] which are associated to a decrease of cellular activation.

We observed that the expression of integrin CD11b, a marker of cell activation that in AIA mice is augmented in all splenic innate immune cells (e.g.: NK cells, DCs, monocytes), is restored to CTRL levels in LM11A-31 treated AIA mice. Integrin CD11b plays an important role during inflammation, by enhancing adhesion, migration, chemotaxis and accumulation of macrophages [189]. Pro-inflammatory stimuli as IFNγ/LPS enhance CD11b surface expression in macrophages while immune suppressive factors, such as IL-4 and TGF-b negatively regulate its expression [181]. The reduction in the production of inflammatory cytokines in AIA-treated mice has also an effect on splenic Th17/Treg ratio that is restored to control levels. An increased percentage of Th17 cells and an imbalance in the Th17/Treg lymphocyte ratio is a typical feature of RA. Th17 and Treg cells exert opposing functions in RA: Th17 cells aggravate RA by secreting IL-17 that induces inflammation and joint destruction while Treg cells facilitate the maintenance of self-tolerance by producing IL-10 and TGF-β [190]. As IL-6 is indispensable for the differentiation of pathological Th17 cells and inhibits the differentiation of Tregs [191], the observed changes in LM11A-31 treated AIA mice of decreased Th17 percentage and reduced Th17/Treg ratio are in agreement with the reduction of IL-6 mRNA levels induced by p75NTR inhibition. A reduction of IL-17 is also suggested by the observed decrease in the number of osteoclasts in the knee of AIA-treated mice. It is well known that IL-17 promotes human osteoclastogenesis *in vitro* via the RANK–RANKL system or TNF-α. IL-17 induces human osteoclastogenesis from human monocytes even in the absence of osteoblastic cells or sRANKL through both inductively expressed TNF-α and constitutively expressed RANKL on human monocytes [192, 193] . More recently, it has been demonstrated that even low concentration IL-17 enhances the autophagic activity of osteoclasts precursors (OCPs) through the ERK/mTOR/Beclin1 pathway during osteoclastogenesis, and further promotes osteoclast differentiation. When p75NTR is inhibited, the observed reduction of inflammatory cytokines influences also IgG production, that is significantly reduced in AIA-treated mice. Considering that the main effect of p75NTR blocking is to reduce IL-6 expression and release, our finding is in agreement with previous studies on IL-6 K.O. mice showing the key role of IL-6 in regulating IgG production in AIA mice [187].

All together our *in vivo* data confirm that high p75NTR expression in the synovia is associated to the inflammatory response and that p75NTR inhibition down regulates immune cell activity, specifically reducing the production of inflammatory cytokines and inflammatory mediators that results in a striking reduction in the clinical signs of the disease.

To better understand whether p75NTR activation is releated to inflammatory activity, we decided to investigate the release of p75ECD in JIA patients. It is known from studies on neurons and neurodegenerative diseases that an increase in p75NTR in tissues is also associated with an increase in p75NTR processing. Cleavage of p75NTR by α-secretase, as ADAM-10 or 17, in the stalk region nearby the transmembrane domain of p75NTR results in the release of the extracellular domain fragment (ECD). The activation of p75NTR occurs through its shedding, with the release of the p75ECD as the first, necessary step that is then followed by the regulated-intramembrane proteolysis of the resulting membrane-bound C-terminal fragment (CTF) by gamma-secretase. We found that this mechanism is active in the inflamed synovia of JIA and RA patients since ECD accumulates in the synovial fluids of patients in the active phase of the disease when p75NTR is extremely highly expressed in the synovial tissue and in the infiltrating immune cells. This is in agreement with the reported accumulation of ECD in the tissues and biological fluids of patients with neurodegenerative diseases. To better characterize how p75NTR shedding is regulated during arthrtis inflammatory response, we analyzed *in vitro* synoviocyte response to IL-1β. As IL-1β strongly induces p75NTR expression in FLS it is not surprising that high levels of p75ECD were released in the conditioned media. The shedding of p75NTR is regulated by ADAM-17, an α-secretase that is higly expressed in RA- FLS and whose levels are directly under the controll of inflammatory cytokines as IL-1β. Our data indicate that IL-1β enhances the expression of p75NTR in the synovia and contemporary ehances the levels of ADAM-17 that is responsable for p75NTR shedding and further processing. As the shedding of p75NTR is an indication of the receptor activity, the analysis of p75ECD in the tissue and in blood may represent a useful marker to monitoring the inflammatory response in arthritis patients.

We measured p75ECD levels in the synovial fluids and in blood of JIA patients and the analysis of matched samples demonstrated that concentration of p75ECD is higher in the inflamed synovial fluids than in the plasma of the patients, highlightening that its highest concentration is at the site of inflammation rather than in peripheral blood. The levels of p75ECD in JIA plasma were nevertheless higher than the ones in the plasma of healthy donors. In JIA patients the correlation of p75ECD concentration with the levels of pro-inflammatory driven-cytokines in arthritis, showed that the shedding of p75ECD is directly proportional to disease gravity. Moreover, the concentration of p75ECD in plasma of JIA patients is strongly upregulated at the onset of the disease and decreases to almost control levels in patients in remission-on treatment. It is thus reasonable to hypothesize that p75NTR-ECD concentration is strictly connected to the acute phase of inflammation in arthritis patients, while remission status is characterized by very low levels of released ECD. The possibility to use p75ECD as a prognostic marker in JIA patients will be further evaluate in the next future.

In conclusion, taking all our results together we provided new evidence for a novel pathogenetic mechanism in arthritis demonstrating that overexpression of p75NTR amplifies inflammatory responses through activation of intracellular pathways that lead to inflammatory cytokine production. As both the *in vitro* and *in vivo* study suggest, the inhibition of p75NTR strongly down-regulates inflammatory mediator release and dampens immune cell activity. Accumulation of p75ECD resulting from receptor shedding might represent a novel biomarker in JIA patients as p75ECD accumulation in synovial fluids and plasma correlate with inflammatory cytokines production and disease gravity.

A hypothesis worth investigating is that a costituvely high expression of p75NTR might be at the base of the inability of blocking the inflammatory response in not responder arthritis patients that do not reach remission and continue to accumulate damage, with significant social and economic costs. More clinical studies are needed in order to evaluate the possibility to use p75NTR or p75NTR-ECD expression in monitoring the inflammatory state of arthritis patients and the effects of specific treatments on disease duration. Overall, our results represent a strong rational for the targeting of p75NTR as a novel treatment in chronic arthritis.

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