Expression and function of P75 neurotrophin receptor in the immune system

Dissertation

zur Erlangung des Doktorgrades
der Naturwissenschaften (Dr. rer. nat.)
der Fakultät für Biologie
der Ludwig-Maximilians-Universität München

vorgelegt von
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München, August 2018

Hiermit erkläre ich, C.Korcan Ayata, an Eides statt, dass ich die vorliegende Dissertation mit Ausnahme von Tierbehandlung und Immunfluoreszenzfärbung zur Quantifizierung des Keimzentrums selbständig und ohne unerlaubte Hilfe angefertigt habe.

Ich habe weder anderweitig versucht, eine Dissertation oder Teile einer Dissertation einzureichen beziehungsweise einer Prüfungskommission vorzulegen, noch eine Doktorprüfung durchzuführen.

Munich, 09.August.2018

C. Korcan Ayata

Dissertation eingereicht:	09.08.2018
Tag der mündlichen Prüfung:	22.01.2019
Erstgutachter:	Prof. Elisabeth Weiß
Zweitgutachter:	Prof. Bettina Kempkes

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1. Summary

The nerve growth factor family of neurotrophins consists of four well-studied members: Nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4). Neurotrophins are essential for the development, survival and maintenance of the nervous system. There is increasing evidence that neurotrophins and their receptors may have functions in the immune system. Nerve growth factor receptor (NGFR, $p75^{NTR}$, TNFRSF16, CD271), encoded by *NGFR* gene, is the only receptor with the ability to signal in response to all neurotrophins. Although $p75^{NTR}$ has been intensively investigated under neurobiological aspects, little is known about its role in the immune system.

The aim of this study was to quantify the expression of $p75^{NTR}$ in isolated populations of immune cells and in immune organs as well as to learn about its possible functions in the immune system using knockout mice.

We identified, full-length transcripts of *NGFR* in human immune organs and in various immune cell subsets. In adenoids and tonsils *NGFR* gene was expressed comparable to CNS levels. Immunostaining with specific mAbs showed $p75^{NTR}$ on follicular dendritic cells and on a subset of germinal centre B cells. The study was further expanded to investigate human conditions that are known to be prone to formation of extranodal follicles. Expression of $p75^{NTR}$ in extranodal follicles of Hashimoto's Thyroiditis (HT) and Follicular B cell Lymphoma suggests $p75^{NTR}$ mediated functions during autoimmune diseases and malignancy.

Conducting experiments on $p75^{NTR}$ exon 4 deficient animals was the final step of this project. Mice were immunized with three different antigens that served to induce germinal centre reaction, primary and memory response in terms of Ig production. Deletion of $p75^{NTR}$ did not cause any major alterations in immune cell compartments and memory response compared to wild type mice; on the other hand increased production of antigen specific IgM and elevated number of germinal centres in the spleen were observed in deficient animals.

In conclusion the expression of $p75^{NTR}$ in normal lymphatic tissue suggests a role in the adaptive immune system. *In vivo* studies performed with $p75^{NTR}$ exon 4 knockout mice indicate a modulatory role in selection, survival and maturation process of IgM memory B cells and/or IgM plasma cells.

2. Introduction

The fate of cells is genetically programmed and under normal circumstances, each cell follows its program and performs the relevant function as much as the organism needs its contribution. In some special situations, strong effector signals coming from extracellular sources have the ability to initiate gene transcription to promote the cells to a much more capable state in many ways, as required by the organism. These factors and their respective receptors differ from each other among various cell types. Some of these proteins are secreted and can initiate target cells distributed in distant locations, while others need cell-to-cell interaction to exert their functions. Expression profiles of the receptors and ligands are determined by the actual status of the relevant tissue system. These factors may be referred as hormones, growth factors, trophic factors or cytokines depending on their target cell, the tissue they have been secreted or to the compartment where their functional characteristics were initially identified. As scientists go deeper in their investigations, growth factor families become much closer to each other, in some cases sharing receptors or ligands.

In this context p75 neurotrophin receptor ($p75^{NTR}$) could be a good example. It is a low affinity receptor that all members of the nerve growth factor (NGF) family of neurotrophins (NT) can signal through (Radeke et al., 1987; Rodriguez-Tebar et al., 1992); structurally it is a member of tumour necrosis factor receptor superfamily (TNFRSF) (Hempstead and Chao 1989). Moreover, $p75^{NTR}$ has the ability to form receptor complexes with many different membrane proteins such as tropomyosin receptor kinase (Trk), Nogo and Sortilin receptors, leading to formation of high affinity targets for various ligands. There are many examples where a well-known growth factor turns out to be used or produced also by many other cell types. NGF itself can be considered a good example. It is synthesized in the hypothalamus, pituitary gland, thyroid gland, testes, and the epididymis, moreover various cell types including vascular smooth muscle cells, fibroblasts and some immune cells, also express it (Calzà et al., 1997).

The vast majority of neuronal cell populations in the mammalian brain are formed prenatally, but still, some parts of the adult brain have the ability to regenerate by developing new neurons from neural stem cells. NTs are the key

factors that help to stimulate and control neurogenesis. Currently, in mammals, the NGF family of neurotrophic factors consist of four members: NGF (Levi-Montalcini, 1987), BDNF (Brain derived neurotrophic factor) (Barde et al., 1982; Leibrock et al., 1989), NT-3 (Neurotrophin 3) (Ernfors et al., 1990; Hohn et al., 1990; Maisonpierre et al., 1990, Rosenthal et al., 1990) and NT-4/5 (Neurotrophin 4/5) (Berkemeier et al., 1991). NTs are synthesized as precursor proteins, then modified to mature forms and secreted. NTs form dimers to act through neurotrophin receptors. In the mature protein, half of the amino acid residues are common to all neurotrophins and they all share six cysteine residues at identical positions. This leads to the formation of the disulfide bridges and the similar tertiary structure of the molecules. The neurotrophins are able to form stable heterodimers *in vitro* (Jungbluth et al., 1994), but *in vivo* role of these NT heterodimers is not known.

The NGF family of neurotrophins has been shown to modulate different functions in the nervous system. The main tasks of the NTs are: enhanced survival of neurons, development and maintenance of central and peripheral nervous system regulating the balanced release of neurotransmitters, dendrite growth and axonal regeneration after injury (Levi-Montalcini, 1987; Sendtner et al., 1992; Thoenen, 1995; Lewin and Barde, 1996; Sawai et al., 1996; Barde, 1997; Semkova and Krieglstein, 1999).

2.1 Neurotrophic factors

2.1.1 Nerve growth factor (NGF)

NGF is the member that the whole family was named after. It was identified more than a half century ago (Levi-Montalcini, 1952). The essential role of NGF is neuronal survival and development (Levi-Montalcini, 1987). NGF performs many other roles in the nervous system such as repair, regeneration, maintenance and protection of sympathetic and sensory neurons. Mice lacking a functional NGF gene do not possess small diameter sensory neurons and sympathetic postganglionic neurons and die shortly after birth (Crowley et al., 1994). Newborn animals injected with anti-NGF antibodies, loose permanently the majority of sympathetic ganglion cells (Goedert et al., 1978). NGF is a 130-140-kDa complex composed of α, β and γ subunits (Bax et al., 1993). The biological activity is due to the β subunit (Fahnestock, 1991); therefore the name βNGF is being used for this protein. Two β subunits

homodimerize to form the 26-kDa-polypeptide dimer (Ibanez, 1998; McDonald et al., 1991). The βNGF gene is located on chromosome 1 in humans (Francke et al., 1983) and encodes a 34-kDa precursor also called pre-proNGF. An initial cleavage forms proNGF. A secondary enzymatic process by a furin or pro-convertase, leads to the formation of the mature and biologically active βNGF (Fahnestock, 1991; Chao, 2003). Human and mouse proteins show 90% homology (Ullrich et al., 1983).

NGF can signal through both TRKA and $p75^{NTR}$ that translate into activation of various signaling pathways depending on the TRKA-$p75^{NTR}$ ratio.

2.1.2 Brain derived neurotrophic factor (BDNF)

BDNF is the second member of the NGF family. As its name indicates, BDNF was originally found in brain tissue (Yves-Alain Barde, 1982). BDNF exerts its function mostly on certain neurons of the central nervous system (CNS) and peripheral nervous system (PNS). BDNF supports the survival of primary sensory neurons that are not responsive to NGF. BDNF has a trophic action on retinal, cholinergic, and dopaminergic neurons, and in the peripheral nervous system it acts on both motor and sensory neurons. BDNF does not only support existing neurons, but also promotes growth and differentiation of newly developing neurons through TrkB and $p75^{NTR}$.

The most obvious phenotype of BDNF knockout mouse is the lack of proper coordination of movement and balance. Morphologically, there is no reduction in size of cochlear ganglia, motor neurons of facial nucleus or lumbar spinal cord. On the contrary the volume of nodose-petrosal complex, vestibular and trigeminal ganglia were found to be reduced. Mice fail to thrive beyond postnatal day 8 and die around 3 weeks of age.

2.1.3 Neurotrophin 3 (NT-3)

NT-3 was discovered due to its high resemblance to NGF and BDNF (Hohn et al., 1990) and it is encoded by *Ntf3* gene. The obvious function of NT-3 is to support the survival and differentiation of existing neurons; additionally it promotes growth and differentiation of newly developing neurons and synapses. Activating TRKC, TRKB and $p75^{NTR}$, NT-3 is considered special because it can potentially act on more target cells than any other NT.

The ligand dependent activation of TRKC by NT-3 prevents the myelination process. During the developmental phase of glial proliferation and elongation, NT-3 levels decrease whereas TRKC and $p75^{NTR}$ remain constant. With the initiation of myelination, NT-3 protein levels drop to undetectable amounts, stopping the inhibitory action (Cosgaya et al., 2002).

Ntf3 deficient mice showed severe movement defects of the limbs, and the majority died shortly after birth (Ernfors et al., 1994). It was observed that considerable portions of peripheral sensory and sympathetic neurons were lost, whereas motor neurons were not affected. A quantitative analysis revealed that the number of muscle spindles in heterozygous mice was half of that of control mice. When NT-3 was inactivated in embryonic stem cells (ESC), homozygous knockouts completely lacked peripheral sense (Tessarollo et al., 1994). In the same model, knockout animals exhibited severe neurological dysfunction. A loss of muscle sensory neurons has been observed in targeted mutation of *Ntf3* in mouse (Klein et al., 1994). Additionally, perinatal death seen in *Ntf3* deficient mice is presumably due to the essential role of NT-3 in heart development (Donovan et al., 1996).

2.1.4 Neurotrophin 4 (NT-4)

NT-4 has not been as extensively studied as the rest of the family. It is encoded by *Ntf4* gene. There are several points about NT-4 that make this neurotrophin look different than others. NT-4 expression is ubiquitous and its modulation seems to be independent from environmental factors (Ip et al., 1992), but dependent on $p75^{NTR}$ for competent signaling (Rydén et al., 1995), as well as neuronal retrograde transport (Curtis et al., 1995).

Ntf4 deficient mice examined so far have only minor cellular deficits (loss of sensory neurons in the nodose-petrosal and geniculate ganglia) and develop normally after birth (Conover et al., 1995; Liu et al., 1995). A detailed examination of the learning and memory functions of *Ntf4* deficient mice with the use of fear conditioning, revealed significant deficits in mutant animals at 2 and 24 hours after training, on the other hand the result for 30 minutes was similar. Experiments on slices prepared from hippocampus revealed a decrease in long-term potentiation (Xie et al., 2000). NT-4 can signal through both TRKB and $p75^{NTR}$.

2.1.5 Pro-Neurotrophins

Members of the NGF family of neurotrophins are processed intracellularly from immature precursors called proneurotrophins (ProNT). At the initial phase ProNTs have been considered inactive precursors. Further investigation in recent years demonstrated that this is not the exact situation. ProNGF has been identified as the predominant form of NGF in many tissues and it turned out to be a biologically important molecule (Lee et al., 2001; Fahnestock et al., 2001; Pedraza et al., 2005; and Reinshagen et al., 2000).

ProNGF and proBDNF were shown to be cleaved extracellularly, following their secretion. The process is performed by numerous proteolytic enzymes including furin, plasmin and MMPs (Lee et al., 2001).

ProNGF is the best-studied pro-neurotrophin. It is the high-affinity ligand for $p75^{NTR}$-Sortilin receptor complex and it induces apoptosis in neurons (Nykjaer et al., 2004). Moreover, proNGF mediates cell death of oligodendrocytes following spinal cord injury *in vivo* (Beattie et al., 2002). Additionally, proNGF has been suggested to be responsible for the neurodegeneration that occurs in the brains of Alzheimer's disease patients (Pedraza et al., 2005). These new findings have brought an increased complexity to the NT research.

2.2 Neurotrophic factor receptors

$P75^{NTR}$ and Trk receptor family are the two types of receptors responsible for transducing neurotrophin signals. They have different structural components to activate downstream signaling pathways. $P75^{NTR}$ and Trk receptors were denominated as low- and high-affinity receptors as they have dissociation constants of 10^{-9} M and 10^{-11} M respectively for mature NGF (Lewin and Barde, 1996; Friedman and Greene, 1999). Recent studies on "so called" low-affinity receptor $p75^{NTR}$ and high affinity Trk receptors revealed that the presence of both type receptors is required on the same cell for establishment of real high affinity sites for the neurotrophins (Hempstead et al., 1991, Bibel et al., 1999, Roux and Barker, 2002). As a pan-neurotrophin receptor, $p75^{NTR}$ can be triggered by all members of the NGF family (Rodríguez-Tebar et al., 1990, 1992; Hempstead, 2002). On the other hand, each Trk receptor has a preferential ligand that binds with higher affinity.

Another crucial difference between these two receptor types is the lack of a catalytic tyrosine kinase domain in $p75^{NTR}$.

2.2.1 $P75^{NTR}$ the orphan TNF receptor

The $p75^{NTR}$ is the first receptor that has been identified as NGF receptor. It is a 75-kDa glycoprotein (Huebner et al., 1986) and has four cysteine-rich regions (CRR) in the extra cellular domain (ECD). $P75^{NTR}$ belongs to the family of TNF receptors, which contains many receptors as CD40, OX-40, FAS and BAFF-R that are all indispensable for immune system functions. This family of receptors is mainly distinguished with their multiple CRRs for ligand binding, a single pass transmembrane domain and a non-catalytic cytoplasmic domain (Locksley et al., 2003). Although being a member of TNFR superfamily, a TNF ligand for $p75^{NTR}$ has not been identified so far. The sequences of four CRRs are responsible for the negatively charged structure and for binding of all neurotrophins of the NGF family to the receptor (Chapman and Kuntz, 1995), with similar affinities (Rodríguez-Tebar et al., 1990, 1992; Hempstead, 2002).

$P75^{NTR}$ protein has three major domains that interact with ligands, co-receptors or adapter proteins: Neurotrophin binding domain, intracellular juxta-membrane domain and death domain. The neurotrophin-binding domain is composed of three cysteine rich regions coded by exon 3. Both intracellular juxta-membrane domains coded by exon 5 and death domain coded by exon 6 play important roles to substitute for the lack of a catalytic domain. They provide binding sites for recruiting adapter proteins required for downstream signaling. There is only one transcript variant reported so far, which lacks exon 3, coding for the neurotrophin binding domain. This short isoform is not able to bind to neurotrophins but can interact with Trk receptors. It is called s-p75 and had only been reported to exist in mice (von Schack et al., 2001, Ibanez, 2002). Neurotrophins and Trk receptors always form dimeric molecules whereas $p75^{NTR}$ can form dimers as well as trimers similar to other members of the TNF receptor superfamily (Anastasia et al., 2015).

ProNGF binds to $p75^{NTR}$ with high affinity and induces apoptosis (Lee et al., 2001, Beattie et al., 2002). A 95-kDa co-receptor called Sortilin (encoded by *SORT1* gene) is required for induction of apoptosis by formation of a high affinity receptor complex with $p75^{NTR}$ (Nykjaer et al., 2004). This apoptotic effect mediated by the

$p75^{NTR}$ – Sortilin complex and proNGF is not unique; proBDNF leads to similar consequences (Teng et al., 2005). Additional to $p75^{NTR}$ - Trk and NT-proNT ratios, the presence or absence of sortilin on the same cell that expresses $p75^{NTR}$ will also determine cell fate. In fact, the inhibition of sortilin, specifically in $p75^{NTR}$ expressing neurons, suppresses proNT mediated apoptotic action (Lu et al., 2005).

Activation of the $p75^{NTR}$ by NTs and proNTs induces recruitment of several adapter proteins that promote cell survival as well as apoptosis (Hempstead and Salzer, 2002), these adapter proteins are widely shared among other members of TNFR superfamily. TRAF6, NRIF, NRAGE, NADE, IRAK and SC-1 have been identified as adapter proteins interacting with intracellular domains of $p75^{NTR}$ (Fig. 2.2, reviewed by Arévalo and Wu 2006).

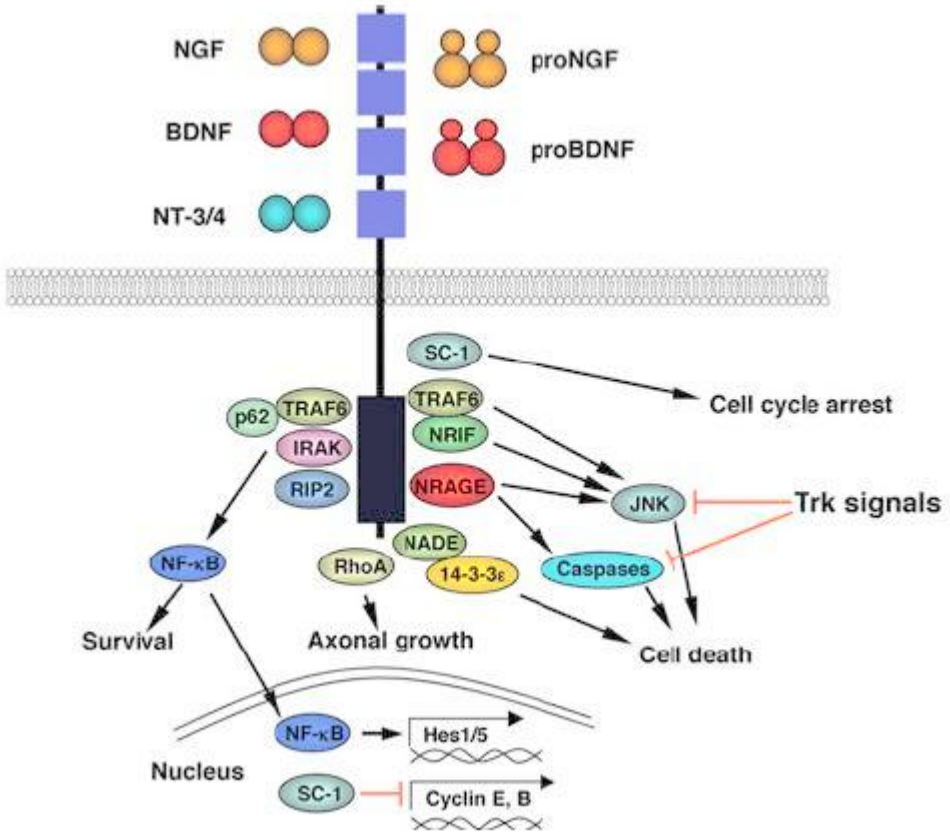

Figure 2.2

Overview of neurotrophin or pro-neurotrophin mediated signaling through $p75^{NTR}$. Many adapter proteins can be recruited to the p75 intracellular domain. TRAF6, NRIF, NRAGE, NADE, IRAK and SC-1 can interact with p75-ICD. Initiation of various pathways through different adapter molecules results in a range of effects including axonal growth, growth cone collapse, survival, and apoptosis or cell cycle arrest. Trk receptor signaling may block cell death signaling through $p75^{NTR}$. Modified from Arévalo and Wu 2006.

TNF ligands and receptors are processed by proteases, yielding soluble effector proteins or intracellular signaling elements. As being a member of TNFR superfamily, $p75^{NTR}$ carries a similar feature. A ligand independent α-secretase cleavage of $p75^{NTR}$ leads to a soluble p75 fraction called p75ECD (extracellular domain) and a membrane bound fraction called p75ICD (intracellular domain) (DiStefano and Johnson, 1988; Zupan et al., 1989; Barker et al., 1991; DiStefano et al., 1993). Accumulation of p75ICD promotes cell death. A consecutive γ-secretase cleavage of p75ICD yields an intracellular fragment with nuclear functions (Kanning et al., 2003). α-secretase or γ-secretase cleavage of $p75^{NTR}$ activates different intracellular signaling pathways (Vicario et al., 2015). The α-secretase enzyme cleaving $p75^{NTR}$ is called TACE (Tumour necrosis factor α converting enzyme). There are a number of studies reporting that interaction of $p75^{NTR}$ with other proteins like sortilin (Skjeldal et al., 2012), Trk receptors (Ceni et al., 2010) and myelin associated ligands (Domeniconi et al., 2005) influence its proteolytic cleavage. Recent research showed that the ectodomain of $p75^{NTR}$ has neuroprotective role against amyloid-beta plaque toxicity during Alzheimer's disease (Yao X-Q et al., 2015).

Modulation of axonal growth depends on different ligands and adapter proteins that interact with $p75^{NTR}$. While neurotrophin signaling promotes axonal growth, myelin-derived growth inhibitors lead to growth cone collapse. Neurotrophins have also roles on regulation of RhoA, which has the ability to manage the organization of the actin cytoskeleton in many cell types (Jaffe and Hall, 2005). Tuning of the axonal growth through $p75^{NTR}$-RhoA pathway is not restricted to neurotrophins. A triple receptor complex formed by $p75^{NTR}$, sortilin and NGR (Nogo receptor, encoded by *RTN4R* gene) is also responsible for mediating inhibitory signals for axonal outgrowth. Identified ligands for this complex are myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp) (Wang et al., 2002; Wong et al., 2002; Mi et al., 2004). It has also been shown that functional LINGO1-$p75^{NTR}$-NGR complexes formed in the plasma membrane and LINGO1 interacts with intracellular membrane compartments of $p75^{NTR}$ (Meabon et al., 2015).

Recently, several studies linked $p75^{NTR}$ expression to cancer stem cells (Dirks F. 2010). It was reported that NGF/proNGF/$p75^{NTR}$ signaling plays a critical role in cancer stem cell self-renewal and plasticity regulation in breast cancer (Tomellini et al., 2015) and melanoma-initiating cells express $p75^{NTR}$ (Boiko et al., 2010).

Moreover, $p75^{NTR}$ is shown to be a marker for tumor-initiating cells in squamous cell carcinoma (Murillo-Sauca 2014).

There is a wide range of functions that have been associated to $p75^{NTR}$, but the complete signaling pathways and complexity of the involving adapter proteins stand as difficult task to solve for the scientists.

$P75^{NTR}$ proves itself to be a unique receptor in many ways:

- Although being a member of the TNFR superfamily, there is no TNF ligand associated with $p75^{NTR}$ yet.
- $P75^{NTR}$ can signal in the presence of all four known neurotrophins.
- $P75^{NTR}$ and sortilin form a high affinity receptor complex for proNTs.
- $P75^{NTR}$ undergoes α– and γ-secretase mediated shedding, resulting in bioactive intracellular fragments.
- $P75^{NTR}$ forms a receptor complex with NGR and LINGO1 for myelin-derived growth inhibitors like myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp).
- $P75^{NTR}$ is a stem cell marker for a variety of cancers.
- Diversity in functions:
    - Promote survival – Induce apoptosis
    - Neurite outgrow – Growth cone collapse
    - Mediate differentiation
    - Enhance proliferation
    - Facilitate myelination

    (Cosgaya et al., 2002, Yamauchi et al., 2004, Du et al., 2006)

2.2.2 Trk family of neurotrophin receptors

The protein tyrosine kinase Trk receptors TRKA, TRKB and TRKC act as specific, high-affinity neurotrophin receptors. Each of the Trk family members shows a preferential ligand binding ability to NTs (Ip et al., 1993). TRKA is the preferred receptor for NGF (Kaplan et al., 1991; Klein et al., 1991), but has a lower affinity for NT-3 or NT4/5. Both BDNF and NT-4 can bind to TRKB. TRKC has a unique ligand, NT-3 (Lamballe et al., 1991), but on the other hand NT-3 can also bind to TRKB with a low affinity (Klein et al., 1991; Ip et al., 1992) (Fig. 2.1).

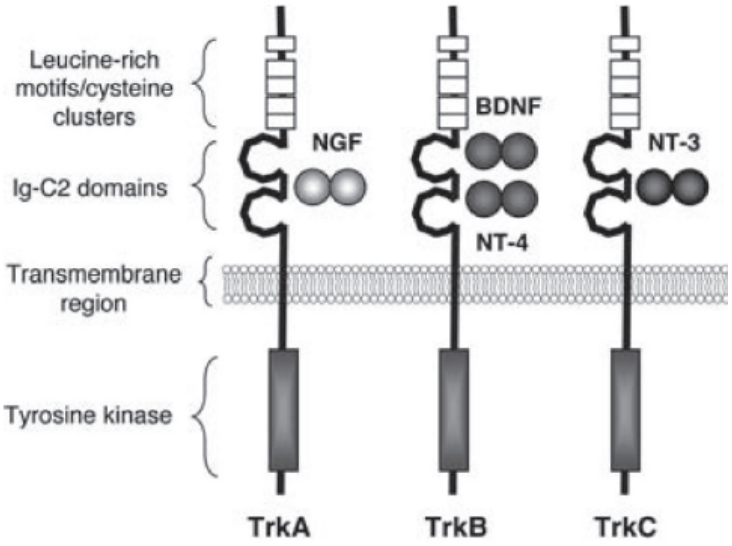

Figure 2.1

Overview of Trk receptors and their preferential neurotrophin ligands.
Trk receptors have three leucine-rich regions (LRR) and two Ig like domains mapped in the extracellular part. The tyrosine kinase domain is located in the intracellular section. Trk Family members share around 50% of their amino acid sequence. NGF selectively binds to TrkA, BDNF and NT-4 to TrkB, finally NT-3 to TrkC. Modified from Arévalo and Wu 2006.

All three Trk receptors harbour a cytosolic domain whose tyrosine-kinase activity is essential for the signal transduction. On the extracellular part they carry three leucine rich regions (LRR) and two Ig like C2 type domains (Ig-C2). They have a single pass trans-membrane domain coupled with a kinase domain. Neurotrophin binding occurs through the Ig-C2 domains (Perez et al., 1995). In situations when there is no interaction with a neurotrophin, the Ig-C2 domains prevent dimerization of the Trk receptors and auto-phosphorylation of the tyrosine kinases (Arevalo et al., 2000). Trk receptors carry structural similarities to each other; they have around 50% amino acid homology.

The main components of Trk receptor downstream signaling pathway are phospholipase C-gamma (PLC-γ), phosphodityl-insitol 3-kinase (PI3-kinase) and SHC adapter proteins. PLC-γ catalyzes the hydrolysis of membrane phospholipids to generate inositol triphosphate (IP3), which is responsible of activating pathways to induce changes in intracellular Ca^{2+}, pH, cytoskeletal functions and gene transcription. PI3-kinase produces phosphoinositides that activate AKT protein kinase, which results in growth factor mediated cell survival. SHC adapter proteins, when phosphorylated by tyrosine kinases, activate RAS and MAP-kinase pathways that in turn activate nuclear transcription factors.

2.2.2.a TRKA

The TrkA receptor is encoded by *NTRK1* gene located on chromosome 1 (Martin-Zanca et al., 1986). It is a 140-kDa trans-membrane protein possessing a tyrosine-kinase domain in the intracellular part. Another key domain of TRKA is the NT binding domain that harbors two Ig-like domains that are involved in NGF selectivity and binding (Wiesmann et al., 1999). The expression profile of the TRKA receptor is widely spread throughout the nervous system (Muragaki et al., 1995).

Receptor homo-dimerization is required for tyrosine kinase activation; this occurs when NGF binds to the receptor, initiating transphosphorylation (Kaplan et al., 1991). This activation involves a group of proteins playing key roles for the cellular functions: RAS, phospholipase C (PLC), protein kinases C (PKC) and phosphatidyl-inositol-3 kinase (PI3) (Obermeier et al., 1993a,b; Melamed et al., 1999; York et al., 2000). The mitogen-activated protein kinase (MAPK) pathways are activated next (Kaplan and Miller, 1997). NGF activation of the TrkA receptor inhibits apoptosis and induces cell proliferation, differentiation and survival (Levi-Montalcini, 1987; Levi Montalcini et al., 1995; Aloe et al., 1997; Bonini et al., 2002).

2.2.2.b TRKB

Identification of TRKB was based on its similarity to TRKA. Human TRKB is encoded by *NTRK2* gene that consists of 24 exons (Yeo et al., 2004). TRKB protein is a single pass transmembrane protein of 822 amino acid residues and the peptide sequence is found to be 49% homologue to TRKA and 55% to TRKC. TRKB is known as high affinity receptor for BDNF and both together they regulate short-term synaptic functions and long-term potentiation of brain synapses. There are three TRKB isoforms in mammals. The full-length TRKB has the typical tyrosine kinase activity, which can transduce BDNF and NT-3 signals to a lesser degree. The two other truncated isoforms are sharing the same extracellular and trans-membrane domains but showing differences in the intracellular part. These isoforms are named as TK-T1 and TK-T2. Additional to splice isoforms, there are also TRKB extracellular deletions, leading decreased responsiveness to BDNF and restriction of NT-3 and NT-4 activation (Kristen et al., 1999).

The first generation of a *Ntrk2* deficient mouse allowed scientists to observe neuronal deficiencies in central and peripheral nervous systems (Klein et al., 1993). Observations were limited to prenatal period and postnatal day 1, which is the point the animals die. Although this knockout served well for understanding the TRKB receptor functions, its impact during postnatal neuronal development was still an open question. The Cre-loxP recombination technique has been used to generate animals that lack TRKB expression in the forebrain (Minichiello et al., 1999). These mice were viable and did not show major morphologic alterations. They were, however, highly impaired in learning under stress conditions (Minichiello et al., 1999). In following years, animal models with disrupted phospholipase C docking site or SHC domain helped better understanding the TRKB functions. PLCγ disruption caused weakened hippocampal long-term potentiation (LTP). In case of SHC disruption, the process had no effect on LTP, but a reduction of MAPK activation was observed on those neurons that had been stimulated with BDNF (Minichiello et al., 2002).

2.2.2.c TRKC

TRKC is coded by *NTRK3* gene and it is the third member of the Trk family is preferentially expressed in the brain (Lamballe et al., 1991), mRNA specific hybridisation showed distributed expression in the hippocampus, cerebral cortex, and the granular cell layer of the cerebellum. TRKC protein has a molecular weigh of 145-kDa. TRKC is the high affinity receptor for NT-3 and does not engage with NGF or BDNF.

Schwann cell function during the development and regeneration of the peripheral nervous system is highly dependent on neurotrophic factor signaling. Although neurotrophins are known to be the key mediators for myelination, abundance of various neurotrophins and the distribution of neurotrophin receptors are crucial for proper myelination. In this context TRKC-NT3 signaling has rather an inhibitory effect whereas $p75^{NTR}$-BDNF enhances myelination (Cosgaya et al., 2002).

2.3 *Ngfr* knockout mice

Two different knockout mouse models for $p75^{NTR}$ have been developed so far. The first model is generated by deletion of exon 3 of *Ngfr,* coding for the three cystein-rich regions forming the neurotrophin-binding domain (Lee et al., 1992). The exon 3 knockout mice still express a p75 protein variant (s-p75) consisting of one cysteine-rich domain, stalk domain, transmembrane domain and intracellular domain. Intensive PCR analysis performed on human, mouse, rat and chicken revealed that s-p75 is conserved across species. Primary Schwann cells obtained from $p75^{NTR}$ exon 3 knockout mice still express the s-p75 variant that cannot bind neurotrophins but interacts with Trk receptors (von Schack et al., 2001).

Another $p75^{NTR}$ knockout mouse has been generated by targeting exon 4, therefore it lacks the transmembrane domain (von Schack et al., 2001). In 2004, Paul et al. reported that a functional fragment of $p75^{NTR}$ 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 (Paul et al., 2004). Both $p75^{NTR}$ exon 3 and exon 4 knockout mice are suitable to investigate NT dependent functions of $p75^{NTR}$ but there is still a need for a knockout model for the investigation of NT independent functions.

2.3.1. $P75^{NTR}$ exon 3 knockout mouse

$P75^{NTR}$ exon 3 KO mice (Lee et al., 1992) 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 translated to 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; the cross-section of the sciatic nerve is 40% smaller.

2.3.2. $P75^{NTR}$ exon 4 knockout mouse

$P75^{NTR}$ exon 4 KO mice (von Schack et al., 2001) were generated after the discovery of s-p75, in order to achieve a complete knockout model. 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.

2.4 Neurotrophic factors and the immune system

In parallel to the identification of the complete family of neurotrophins and receptors, an intense scientific work was allocated to solve the functional puzzle of this growth factor family and their receptors. Secreted neurotrophins also play important roles during sympathetic innervation. In this context NGF is shown to take part in the innervation of the lymphoid organs (Kannan et al., 1994, 1996). Cells expressing NTs and NTRs were partially mapped. In the immune system NTs and NTRs were prominently expressed in some cell subsets of primary and secondary lymphoid organs (Morgan et. al., 1989; Ciriaco et al., 1996; Aloe et al., 1999; Hannestad et al., 1995, 1997).

2.4.1 NGF

NGF is constitutively expressed in the thymus; mostly in the medulla (Katoh-Semba et al., 1993; Aloe et al., 1997; Turrini et al., 2001) and a role for NGF through $p75^{NTR}$ and TrkA during the T cell development in the thymus has been suggested (Marinova et al., 2003). NGF increased the transcription of IL-6 in thymic epithelial cells (Screpanti et al., 1992).

Mast cells (Leon et al., 1994) and T cells have been shown to produce NGF (Ehrhard et al., 1993; Santambrogio et al., 1994; Lambiase et al., 1997), but the level of NGF expression is highly dependent on T cell activation (Ehrhard et al., 1993, Moalem et al., 2000). Both $CD4^+$ and $CD8^+$ populations produce NGF. The level of NGF expression is reported to increase following antigen stimulation, especially in the Th2 subset (Santambrogio et al., 1994, Van Eden et al., 2002).

NGF has also ability to increase survival of memory B cells (Rubartelli et al., 1996) and has been reported to rescue B cells from experimentally induced apoptosis (Kronfeld et al., 2002, Torcia et al., 1996 and 2001). Similarly, NGF

increases survival of macrophages (Garaci et al., 1999) and monocytes (la Sala et al., 2000). Moreover, NGF has been shown to synergetically act with granulocyte-macrophage colony stimulating factor (GM-CSF) to promote human basophilic cell differentiation (Tsuda et al 1991) and to support hemopoietic stem cell development (Chevalier et al., 1994, Auffray et al., 1996). In the mouse, NGF acted as a chemotactic agent on macrophages (Kobayashi et al., 2001) and mast cells (Sawada et al., 2000)

NGF has been considered as a potential biomarker following several studies that were performed in this context. An increased serum level of NGF is found to be quite common during allergic diseases (Frossard et al., 2004), autoimmune disorders (Aloe and Tuveri. 1997, Arredondo et al., 2001), other inflammatory cases (Stanisz M. and Stanisz J. 2000) and stress (Aloe et al., 1994).

Another cell type that is responsive to NGF are bone marrow stromal cells (Rezaee et al., 2010). It is reported that they increase IL-6 expression by activation of ERK and p38 MAPK, but not NFκB.

Recently, it has been shown that NGF attenuates the inflammatory response in LPS activated monocytes by inhibiting production of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α and by inducing IL-10 and IL-1Ra through TRKA receptor (Prencipe et al., 2014).

2.4.2 BDNF

Within the immune system, the presence of BDNF has been first shown in the thymus (Laurenzi et al., 1994; Yamamoto et al., 1996; Timmusk et al., 1999). The main source of BDNF was found to be the stromal cells (Maroder et al., 1996). TrkB receptor expression was reported on immature thymocytes (Maroder et al., 1996). Both $CD4^+$ and $CD8^+$ T cells were reported to transcribe BDNF mRNA and to produce biologically active BDNF (Braun et al., 1999; Kerschensteiner et al., 1999). BDNF was reported to modulate cytokine expression of PBMCs through Trk receptors. Additionally antigen specific T cells had a change in the expression pattern of IL-4, TGFβ, TNFα and INFβ (Bayas et al., 2003).

Recently, a developmental block in the Pre-BII stage in B cells has been reported in *BDNF* knockout mouse (Schuhmann et al., 2005), leading to significant reduction of the B cells in the bone marrow, spleen and blood.

Moreover, BDNF have found to be produced and used by thymic epithelial cells (TEC) and immune cells in the thymus (Berzi et al., 2008), suggesting a role for T cell proliferation and TEC turnover.

2.4.3 NT-3 and NT-4

Compared NGF and BDNF, NT-3 and NT-4 were not as deeply investigated in the immune system. Both NT-3 and NT-4 were reported to exist at both mRNA and protein level in the thymus (Laurenzi et al., 1994, Timmusk et al., 1993, Katoh-Semba et al., 1996). Additionally, NT-3 protein has been detected in the spleen (Zhou & Rush, 1993; Katoh-Semba et al., 1996). *NTF3* and *NTF4* transcripts were reported in $CD4^+$ and $CD8^+$ T cells (Moalem et al., 2000). At protein level, B cells were shown to produce NT-3 (Besser and Wank, 1999).

2.4.4 $P75^{NTR}$

As being able to conduct signals in the presence of all of the NTs and pro-NTs, $p75^{NTR}$ can be considered as an important marker for cells that could respond to NTs. In the thymus, $p75^{NTR}$ was found to be expressed by dendritic cells (Parrens et al., 1999; García-Suárez et al., 2001) and by medullary epithelial cells (Lomen-Hoerth & Shooter, 1995).

The spleen shows a rather compartmentalized expression of $p75^{NTR}$ in follicles (Labouyrie et al., 1997). In human tonsils and adenoids, $p75^{NTR}$ is also present in lymphoid follicles, mainly expressed by follicular dendritic cells (FDC) and lymphocytes (Chesa et al., 1988; Brodie and Gelfand, 1992; Pezzati et al., 1992). Additionally, periarteriolar macrophages and dendritic cells are reported to be positive for $p75^{NTR}$ (Pezzati et al., 1992; Bull et al., 1998; García-Suárez et al., 1997; Labouyrie et al., 1997). $P75^{NTR}$ expression was also located in blood vessels, in the Schwann cells of nerve endings and in the basal epithelium of the mucosa (Esteban et. al. 1995).

The expression of $p75^{NTR}$ by T cells (Kittur et al., 1992; Ehrhard et al., 1993) and by B cells is controversial (Brodie et al., 1996, Schenone et al., 1996). The expression pattern for the T cell and B cell lineages under different activation conditions has never been completely elaborated. There are many conflicting reports

about the expression of NTs and NTRs, mainly due to the variations between targeted cell population and their stage of activation or maturation.

In 2008, Berzi and colleagues revealed that $p75^{NTR}$ is expressed by thymic B cells and thymic epithelial cells (TEC) in healthy adult human thymus. In the same study, $p75^{NTR}$ and BDNF were reported to be colocalized on the proliferating B cells in the germinal centre of the hyperplastic thymus.

Recently it has been reported that plasmocytoid dendritic cells express $p75^{NTR}$ but not Trk receptors (Bandola et al., 2017) and demonstrated $p75^{NTR}$ - NGF driven regulation of T cell priming and cytokine production.

2.4.5 Trk Receptors

Trk receptors were discovered shortly after $p75^{NTR}$ and this finding initiated a massive research concerning their functions in different organ systems. The discovery of the thymus specific splice variants of TrkA (Dubus et al., 2000) strengthened the idea of NTs having broader functions in the thymus.

Transcripts of full-length (Laurenzi et al., 1994; García-Suárez et al., 2002) and truncated isoforms of *Ntrk2* have been reported in the thymus (Lomen-Hoerth & Shooter, 1995). TRKB protein been detected in thymocytes (Maroder et al., 1996; Besser & Wank, 1999; García- Suárez et al., 2002), in stromal cells and in macrophages (García-Suárez et al., 1998, 2002, Levanti et al., 2001, Rezaee et al., 2010). On the other hand, protein expression of TRKC has never been reported.

In the human spleen, TRKA and TRKB were reported to be expressed, mainly by the FDCs (Labouyrie et al., 1997). TRKB was also detected in the splenic macrophages (Shibayama & Koizumi, 1996).

In peripheral blood, mainly T cell populations express TRKB, both isoforms have been detected on $CD4^+$ T cells (Besser & Wank, 1999) and $CD3^+$ T cells (Berzi et al., 2008). Similar to the NT production, TRKB expression level depends on the different developmental stages of T cell populations (Maroder et al., 1996, 2000). TrkA expression in B cells is controversial, it has been reported to exist in B cells (Melamed et al., 1996; Torcia et al., 1996; D'Onofrio et al., 2000), but on the other hand Schenone et al., (1996) stated that neither mRNA, nor protein for TRKA exists in B cells. Recent studies confirm the TRKB expression on B cells (Besser & Wank 1999, D'Onofrio et al., 2000, Berzi et al., 2008). TRKA has been detected on

monocytes and LPS activation induces its expression (Ehrhard et al., 1993a, 1993b; Prencipe et al., 2014). On the other hand, TRKA expression is found to be down regulated during differentiation to macrophages *in-vitro* (Ehrhard et al., 1993).

In summary, the NGF-TRKA pair, as being the most studied receptor-ligand pair, might function in the immune system. Although there are examples of functions concerning both T and B cell biology, each different immune subset seems to bear a unique set of NTs and their receptors.

3. Objectives

This study was undertaken with the following objectives.

1. To identify immune cell subsets expressing $p75^{NTR}$ in blood and immune organs and to determine whether $p75^{NTR}$ expression changes in response to immune cell activation/maturation.

2. To investigate $p75^{NTR}$ in follicular B cell lymphoma and in extranodal lymphoid follicles occurring in autoimmune Hashimoto's Thyroiditis.

3. To learn about a possible role of $p75^{NTR}$ in the regulation of humoral immunity by studying *p75*^{NTR} exon 4 knockout mice.

4. Material and Methods

4.1 Materials

4.1.1 Buffers and reagents

a. Main buffers

Phosphate buffered saline (1X PBS), pH 7.4

9.1 mM Na_2HPO_4

1.7 mM NaH_2PO_4

150 mM NaCl

Tris buffered saline (1X TBS)

10 mM Tris-HCl, pH 8.0

150 mM NaCl

b. ELISA buffers

ELISA coating buffer, pH 9.6

15 mM Na_2CO_3

35 mM $NaHCO_3$

ELISA washing buffer

1X PBS + 0.05% Tween-20

ELISA blocking buffer

1X PBS + 2-10% FCS

ELISA substrate buffers

1- 20 mM Na_2HPO_4 +10 mM $C_6H_8O_7$ (Cirtic acid)
Immediately before use, add 1/1000 v/v 3% H_2O_2 and 1-2 g OPD (O-Phenylenediamine), pH 4 – 4,5. Read at 492 nm

2- TMB (Tetra-methyl-benzidine) substrate solution (Sigma)
Filters: Read 450 nm, reference 550 nm

ELISA stop solution

1 or 2 M H_2SO_4

c. RNA-DNA-PCR buffers

TAE Buffer (Tris-Acetate-EDTA) 50x

242 g Tris base

57.1 ml Acetic acid

100 ml 0.5 M EDTA

Add distilled water to 1 liter and adjust pH to 8.5

Lysis Buffer: RNA extraction from FFPE tissue

1 M Tris-HCl

0.5 M EDTA

10% SDS

pH 8

Add 20 mg/ml Proteinase K (Sigma), (Add fresh before use)

Tail digestion buffer

100 mM Tris-HCl, pH 8.5

200 mM NaCl

5 mM EDTA

0.2% SDS

100 μg/ml Proteinase K (Sigma), (Add fresh before use)

d. FACS buffers/reagents

FACS buffer (staining/washing)

1X PBS + 2% heat-inactivated FCS

Cell permeabilization buffer

1X PBS + 1% heat-inactivated FCS + 0.1% (w/v) saponin (Sigma), pH 7.4

Propidium Iodide solution

1 mg/ml in 1X PBS

e. Immuno-staining buffers

Cell/Tissue section fixing solution - 4% PFA

Paraformaldehyde 4% (w/v) in 1X PBS, pH 7.4

Endogenous peroxidase activity stopping solution

3% H_2O_2 in methanol

Blocking buffer

1X PBS + 10% heat-inactivated FCS + 5% serum from secondary antibody host animal.

f. Cell culture reagents

Cell culture medium

RPMI 1640 (Gibco)

1% Pen-strep (Penicillin G 10,000 units/ml; streptomycin 10,000 µg/ml; Gibco)

5% or 10% heat-inactivated fetal calf serum (FCS)

Red blood cells (RBC) lysis buffer

0.83% ammonium chloride in PBS or RBC lysis solution (Qiagen)

4.1.2 Antibodies

Antibodies (anti-human) for FACS and immunohistochemistry

Target (Human)	Manufacturer	Label	Clone	Method/Concentration
$P75^{NTR}$	BD	None/Biotin	C40-1457	IHC, IF (fz, ffpe)*, 1-10 μg/ml
$P75^{NTR}$	BD	PE	C40-1457	FACS, 2.5 μg/m
$P75^{NTR}$	Sigma	None	ME 20.4	FACS, IHC (fz), 1-1 0μg/ml
FDC	DAKO	None	CNA.42	IHC, IF (fz, ffpe), 10 μg/ml
BDNF	R&D	None	35928	IHC, IF (fz), 10 μg/ml
TRKB	R&D	None	72509	IHC, IF (fz), 10 μg/ml
CD4	BD	FITC/PerCP	RPA-T4	FACS 1/25 – 1/50
CD8	BD	FITC/PerCP	RPA-T8	FACS 1/25 – 1/50
CD14	BD	FITC/PerCP	M5E2	FACS 1/25 – 1/50
CD19	DAKO	FITC	HD37	FACS 1/25 – 1/50
CD20	BD	FITC/PerCP	L27	FACS 1/25 – 1/50
CD21	DAKO	FITC	1F8	IHC, IF (fz, ffpe), 1-10 μg/ml
CD27	BD	FITC	M-T271	FACS 1/50
CD38	BD	FITC	HIT2	FACS 1/50
CD56	BD	None	MY31	FACS 1/25 – 1/50
CD77	BD	FITC	B5B	FACS 1/50
CD79a	DAKO	None	CJB117	IHC, IF (fz, ffpe), 1-10 μg/ml
IgM	BD	FITC	G20-127	FACS 1/25 – 1/50
IgD	BD	FITC	IA6-2	FACS 1/25 – 1/50

Isotype controls

Isotype	Manufacturer	Label	Method/Concentration
Mouse IgG1	BD	FITC/PE/PerCP	FACS 1/25 – 1/50
Mouse IgG2a	BD	FITC/PE/PerCP	FACS 1/25 – 1/50
Mouse IgG2b	BD	FITC/PE/PerCP	FACS 1/25 – 1/50
Mouse IgM	DAKO	None	IHC, IF (fz, ffpe), 1-10 μg/ml
Mouse IgG1	DAKO	None/Biotin	IHC, IF (fz, ffpe), 1-10 μg/ml

* fz: frozen, ffpe: formalin fixed paraffin embedded, IHC: Immunohistochemistry, IF: Immunolorescence

Antibodies (anti-mouse) for immunohistochemistry

Target (Mouse)	Manufacturer	Label	Clone	Method/Concentration
$P75^{NTR}$	Serotec	None	ab8874	IHC, IF (fz)*, 0.1-1 μg/ml
IgM	Jackson	Cy5	-	IF (fz), 1-10 μg/ml
IgD	eBiosciences	Biotin	11-26c	IF (fz), 1-10 μg/ml
B220	BD	FITC	RA3-6B2	IF (fz), 1-10 μg/ml
FDC	BD	None	FDC-M1	IHC, IF (fz), 10 μg/ml
CD4	Invitrogen	Alexa-488	RM4-5	IF (fz), 1-10 μg/ml
CD8	Invitrogen	Alexa-488	5H10	IF (fz), 1-10 μg/ml

Antibodies (anti-mouse) for ELISA

Target (Mouse)	Manufacturer	Label	Clone	Method/Concentration
IgG1	BD	Biotin	A85-1	ELISA detection 1 μg/ml
IgG1	BD	None	A85-3	ELISA coating 1 μg/ml
IgG2a/2b	BD	Biotin	R2-40	ELISA detection 1 μg/ml
IgG2a	BD	None	R11-89	ELISA coating 1 μg/ml
IgG2b	BD	None	R9-91	ELISA coating 1 μg/ml
IgM	BD	Biotin	R6-60.2	ELISA detection 1 μg/ml
IgM	BD	None	II/41	ELISA coating 1 μg/ml
IgG	Jackson	None	Goat	ELISA detection 1 μg/ml
IgG	Jackson	HRP	Goat	ELISA coating 1 μg/ml
IgM	Jackson	None	Goat	ELISA detection 1 μg/ml
IgM	Jackson	HRP	Goat	ELISA coating 1 μg/ml

* fz: frozen, ffpe: formalin fixed paraffin embedded, IHC: Immunohistochemistry, IF: Immunolorescence

Antibodies (anti-mouse) for FACS

Target (Mouse)	Manufacturer	Label	Clone	Method/Concentration
Specificity	Company	Label	Clone	FACS 0.5 μg/ml
CD11b	Pharmingen	PE	M1/70	FACS 0.5 μg/ml
CD19	Pharmingen	FITC	1D3	FACS 0.5 μg/ml
CD19	Pharmingen	PE	1D3	FACS 0.5 μg/ml
CD45R/B220	Pharmingen	PerCP	RA3-6B2	FACS 0.5 μg/ml
CD8a	Pharmingen	APC	53-6.7	FACS 0.5 μg/ml
CD4	Pharmingen	PerCP	RM4-5	FACS 0.5 μg/ml
CD25	Pharmingen	PE	3C7	FACS 0.5 μg/ml
CD62L	Pharmingen	Biotin	MEL-14	FACS 0.5 μg/ml
IgMb	Pharmingen	FITC, PE	AF6-78	FACS 0.5 μg/ml
MHC Class II I-A/I-E	Pharmingen	Biotin	2G9	FACS 0.5 μg/ml
FAS	Pharmingen	PE	Jo2	FACS 0.5 μg/ml

4.1.3 PRIMERS AND PROBES

a. Conventional PCR primers

Human *GAPDH* - Reference gene (RG).

Forward primer: 5'-GAAGGTGAAGGTCGGAGTC-3'

Reverse primer: 5'-GAAGATGGTGATGGGATTTC-3'

Amplicon length: 326 bp

Human *NGFR* Death domain

Forward primer: 5'-AAAACTCCACAGCGACAGTGGC-3'

Reverse primer: 5'-AGTGGACTCACTGCACAGACTC-3'

Amplicon length: 369 bp

Human *NGFR* Intracellular domains

Forward primer: 5'-GCCTTGTGGCCTACATAGCCTT-3'

Reverse primer: 5'-AGTGGACTCACTGCACAGACTC-3'

Amplicon length: 473 bp

Human *NGFR* Neurotrophin binding and stalk domains

Forward primer: 5'-CCGTGTGTGAGCCCTGCCTGGA-3'

Reverse primer: 5'-AAGGCTATGTAGGCCACAAGGC-3'

Amplicon length: 656 bp

Human *NTRK1*

Forward primer: 5'-ATGTCACCAGTGACCTCAACAG-3'

Reverse primer: 5'-GTGGAGAAGAAGGACGAAACAC-3'

Amplicon length: 485 bp

b. Quantitative TaqMan PCR primers and probes

Human *NGFR*

Forward primer: 5'-CCTGGCTGCTGTGGTTGTG-3'

Reverse primer: 5'-CTGTTGGCTCCTTGCTTGTTCT-3'

Probe: 5'-FAM-CCTACATAGCCTTCAAGAGGTGGAACAGCTG-TAMRA-3'

Human *NTRK1*

Forward primer: 5'- GAAGAGTGGTCTCCGTTTCGTG -3'

Reverse primer: 5'- GAGAGAGACTCCAGAGCGTTGAAG -3'

Probe: 5'-FAM-ACTCCTCGGCTCAGTCGCCTGAATCTCT-TAMRA-3'

Human *NTRK2*

Forward primer: 5'-AGGAGAAGATCAAGATTCTGTCAA-3'

Reverse primer: 5'-GGTCTGAGGTTGGAGATTCG-3'

Probe: 5'-FAM-ACTGTGCATTTTGCACCAACTATCACATTTCT-TAMRA-3'

Human *NTRK3*

Forward primer: 5'-GACAATGGCTTCACCCTGAC-3'

Reverse primer: 5'-CAGCTCAGGCTCCTCCAG-3'

Probe: 5'-FAM-ATGCCAGTGTTGCCCTCACTGTCTACTATC-TAMRA-3'

Human *CD79A*

Forward primer: 5'-CACCAAGAACCGAATCATCACA-3'

Reverse primer: 5'-GAGATGTCCTCATACATGGAGCA-3'

Probe: 5'-FAM-TCGTCCAGGTTCAGGCCTTCATAAAGGTT-TAMRA-3'

Human *GAPDH*

Forward primer: 5'-GAAGGTGAAGGTCGGAGTC-3'

Reverse primer: 5'-GAAGATGGTGATGGGATTTC-3'

Probe: 5'-FAM-CAAGCTTCCCGTTCTCAGCC-TAMRA-3'

PPIA (peptidyl-prolyl isomerase A - cyclophilin A), *GAPDH*, *BDNF*, *SORT1* (Sortilin), *RTN4R* (Nogo Receptor) primers and probes were purchased from Applied Biosystems. Other primers and probes are self designed using Primer3 (Steve Rozen and Helen J. Skaletsky 2000) and Primer Express v1.0 (Applied Biosystems). All primer/probe sequences are controlled for self-complimentarity or possible interactions with each other. Designed primers are tested with conventional PCR experiments to amplify from cDNA but not genomic DNA.

4.2 Methods

4.2.1 Cell preparations

Experiments were performed with freshly isolated human peripheral blood mononuclear cells (PBMCs). $2x10^6$ cells per ml were used for all functional assays.

4.2.2 Cell separations

1. PBMCs were separated into different subtypes as $CD4^+$ and $CD8^+$ T lymphocytes, $CD19^+$ B lymphocytes, $CD56^+$ Natural Killer (NK) cells and $CD14^+$ monocytes using magnetic beads by positive or negative selection (Dynal or Miltenyi Biotec/MACS). The purity of the isolated cell types was between 90-96% for different cell populations as determined by FACS analysis (BD FACSCalibur).
2. Adenoid B cell isolation: Fresh adenoids were cut into 2x2 mm pieces and the pieces were passed through 40 or 70 μm cell strainer (BD Falcon). Mononuclear cells (MNCs) were isolated by density gradient centrifugation (Pancoll, PAN Biotech). B cells were separated using CD19 positive cell isolation kit (Dynal or Miltenyi Biotec/MACS).

4.2.3 Cell activation / stimulation

PBMCs or subpopulations were activated with different mitogens or cytokines to analyze various responses as proliferation, differentiation, modulation of expression pattern of some proteins (cytokines/receptors, CD markers etc.).

T cell activation

1. ConA (Sigma): final concentration 10 μg/ml.
2. PMA + Ionomycin (Sigma) PMA: final concentration 3 ng/ml, Ionomycin: final concentration 300 ng/ml.
3. Anti-CD3 (Dako) 1 μg/ml

B cell activation

1. SAC (Staphylococcus Aurous Cowan 1 antigen) (Calbiochem) Dilution: 1:7500.
2. CD40L (R&D)
3. PWM (Pokeweed mitogen) (Sigma): final concentration 5 μg/ml.
4. LPS (Only for mouse B cells)

Monocyte activation

After 24 hr in culture monocytes were activated as follows.

LPS (Lipopolysaccharides from *E.coli*) (Sigma), final concentration 300 ng/ml.

1. IFN-γ (Interferon-gamma) (Roche), final concentration 100 U/ml.
2. TNF-α (Tumor Necrosis Factor-α) (Roche), final concentration 50-100 U/ml.
3. GM-CSF (Granulocyte–Macrophage Colony Stimulating Factor) (R&D), final concentration 250 ng/ml. Culture 7-9 days for macrophage differentiation.

4.2.4 Cell lines

Cell lines were used as positive controls for protein and mRNA expression of $p75^{NTR}$; they were also used to test functional effects of NTs and ProNTs. Cell lines were cultured in 25 or 75 cm^2 Falcon flasks with 5%-10% FCS (Gibco), in RPMI (Gibco) medium + 1% penicillin (50-100 U/ml)/streptomycin (50-100 µg/ml). Incubator settings were 37°C, 95% humidity, 5% CO_2.

SH-SY5Y - Human neuroblastoma clonal sub-line of the neuroepithelioma cell line SK-N-SH that had been established in 1970 from the bone marrow biopsy of a 4-year-old girl with metastatic neuroblastoma.

SK-N-MC - Human neuroblastoma established from the supraorbital metastasis of a neuroblastoma of a 14-year-old girl in 1971.

REH - Human B cell precursor leukemia established from the peripheral blood of a 15-year-old girl with acute lymphoblastic leukemia.

4.2.5 FACS analysis

For intracellular staining, cells were fixed and permeabilized using cell permeabilization buffer. Dead cells and debris were excluded from the analysis by Via-Probe (BD) or forward/side scatter settings. Data were analysed with CellQuest (BD Biosciences) or FlowJo flow cytometry analysis software (Tree Star, Inc.).

4.2.6 ELISA

ELISAs were used to measure cytokines (IL-6 and MCP-1) produced by immune cells or serum immunoglobulin (IgG1, IgG2a, IgG2b, IgM, IgE, IgA, IgG or total Ig) levels of mice.

4.2.7 RNA Extraction

RNA Extraction from formalin fixed paraffin embedded material using Trizol

- Collect 10 sections each 4 μm thick in a 2 ml RNase free tube
- De-paraffinize: Wash 2x10 min xylene, 100%, 90%, and 70% ethanol 5 min
- Place the tubes (caps open) in heat block 37°C for 10-15 min to evaporate ethanol
- Add 200 μl lysis buffer; incubate overnight (16 hours) at 56°C
- Add 1 ml Trizol (for 200 μl lysis buffer); incubate for 5 min at room temperature (RT)
- Add 200 μl chloroform, shake vigorously for 15 sec and incubate 15 min at RT
- Centrifuge 15 min, 11000 rpm*, at 4°C, take upper aqueous phase in a new tube
- Add 0.5 ml isopropanol and mix well, incubate 10 min at RT
- Centrifuge 10 min, 11000 rpm*, at 4°C
- Remove supernatant, wash pellet with 1 ml 75% ethanol and vortex
- Centrifuge 5 min, 8500 rpm*, at 4°C
- Remove SN, air dry the pellet for 5-10 min
- Add water to dissolve RNA, heat to 56°C if necessary

RNA Extraction from fresh cells or frozen material with Qiagen RNeasy kit

- Add 600 μl buffer RLT for 5-10 million cells or 10-15 sections each 10 μm thick, mix and eliminate visible debris by pipetting, do not vortex.
- Transfer lysate to QIAshredder column spin 14000 rpm*, 2 min at RT
- Add 600 μl 70% ethanol to lysate and mix with pipetting
- Transfer 600 μl of this solution to RNeasy column, spin 10000 rpm*, 20 sec at RT, discard follow-through (dft)
- Transfer remaining 600 μl to RNeasy column; spin 10000 rpm*, 20 sec at RT, dft
- Add 350 μl buffer RW1 to column; spin 10000 rpm*, 20 sec at RT, dft
- Add 80 μl DNase solution (70 μl Buffer RDD + 10 μl DNase) incubate 15 min at RT
- Add 350 μl buffer RW1 to column; spin 10000 rpm*, 20 sec at RT, dft
- Use new collection tube; add 500 μl Buffer RPE, spin 10000 rpm*, 20 sec at RT, dft
- Add 500 μl buffer RPE and spin 10000 rpm*, 2 min at RT, dft
- Use 1.5 ml tube to place the column, add 20-40 μl water directly on membrane and spin 10000 rpm*, 1 min at RT

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* Centrifuge used: Eppendorf 5417R

RNA concentration was determined by measuring the absorption at 260nm and 280nm. A280 is used as a measure of impurity of DNA (A280/A260 should be between 1.8 and 2.0). Spectrometers used: Nanodrop and Eppendorf photometer.

4.2.8 Reverse transcription

Complimentary DNA (cDNA) was synthesized using the reverse transcriptase kit (MML-V, Promega), according to manufacturer's protocol.

4.2.9 PCR (polymerase chain reaction) analysis

The cDNA obtained after reverse transcription reaction is used for PCR. Electrophoresis of PCR products was done using 1.5% agarose gels. For sequencing reactions, DNA amplified by PCR was purified using PCR purification kit (Qiagen) according to the manufacturer's protocol. Sequencing of purified DNA from PCR products were performed by Sequiserve, Martinsried.

4.2.10 Quantitative PCR

Quantitative PCR was performed on the GeneAmp5700 or GeneAmp7300 (Applied Biosystems-ABI) using the qPCR Core kit and UNG (Uracyl-N-glycosylase for carry-over prevention) (both Eurogentec). The reaction volume was 25 μl containing 25 – 50 ng RNA converted to cDNA. For all reactions an annealing temperature of 60ºC has been considered for reverse and forward primers and 70ºC for probes. For detection of target genes, primers and probes were designed and were tested for efficiency, additionally; genomic DNA has been used as a template to confirm that it is not amplified (Refer to primer/probe table for complete list of gene expression assays). *GAPDH* and *PPIA* (Cyclophilin A) were selected as reference genes (RG). Relative quantification were calculated using the formula: $\%RG = 100 \times 2^{(-\Delta Ct)}$, where ΔCt is the differende between cycle threshold values of RG and gene of interest (GOI). Combined standard deviation (Sd) of RG and GOI were calculated using the formula based on Muller et al. (2002).

$Sd = 100 \times (2^{Ct\ RG}/2^{Ct\ GOI}) \times ((LN2 \times Sd\ RG)^2 + (LN2 \times Sd\ GOI)^2)^{1/2}$

4.2.11 Immunohistochemistry

Materials that were used for immunohistochemistry:

Human: - Frozen tonsil, adenoid and spleen.
- Formalin fixed paraffin embedded adenoid, healthy thyroid, Hashimoto's Thyroiditis and follicular B cell lymphoma cases.

Mouse: - Frozen spleen, frozen lymphnode.

For frozen material, Superfrost (Menzel) slides were used. 10μm frozen sections were air dried and fixed with 4% PFA, incubated in Methanol + 3% H_2O_2 to stop endogenous peroxidase activity, washed with 1X PBS, and blocked with 10% FBS + 5% serum from secondary antibody host species in 1X PBS. Sections incubated with primary antibodies for 1 hour at room temperature and washed with 1X PBS. PAP or sABC amplification and DAB development step (all from Dako) performed according to the manufacturer's protocol. Hemalaun (Merck) has been used for nuclear counterstaining. Aqueous mounting medium (Dako) and cover slips (Menzel) used to finish preparation.

For formalin fixed and paraffin embedded material, 4 - 7 μm thick sections were prepared on Superfrost/Plus (Menzel) slides. After deparaffinization step (incubation of sections 2 times for 10 minutes in xylene followed by 5 minutes in 100%, 90%, 70% and 50% ethanol), slides were washed with 1X PBS. For epitope retrieval, slides were kept in 95°C citrate buffer (10 mM, pH 6.0) for 20 minutes. All washing, blocking, antibody incubation and developing steps performed similar to cryosection preparations.

4.2.12 Immunofluorescence – Confocal microscopy

All of the section preparation, fixing, washing, blocking and antibody incubation steps were performed as described above in immunohistochemistry section. As secondary antibodies, goat anti-mouse Alexa488 and Alexa594 (Molecular probes), goat anti-mouse (Fab) CY3 and CY5 (Jackson Labs), streptavidin CY5 (Jackson Labs) were used. The nuclei were counterstained with 1 μg/ml DAPI (Sigma-Aldrich). As isotype control, mouse IgG1 (Dako Cytomation, Sigma-Aldrich) and mouse IgM (Cymbus, Dako Cytomation) were used. Confocal

images were taken with a DM-IRE2 laser scanning microscope (Leica) using Leica confocal software version 2.61, Build 1537.

4.2.13 Laser capture microdissection (LCM)

10 µm cryostat sections from freshly frozen adenoids were mounted on PET-slides (P.A.L.M Microlaser). Slides were dried for 30 min at RT protected from air current or dust and 30 min at 37°C, and stored at -80°C. For LCM, sections were rehydrated with DEPC-PBS for 10 min. In order to visualize lymphoid follicles, nuclear stainings were performed with Hemalaun (Merck) for 5 seconds; slides were rinsed in DEPC water and stored in closed containers with silica-gel (Merck). Dense nuclear stainings of the mantel zone were used to guide the laser beam around follicles (Fig. 4.2). 10 µl RNase free filter tips were used to collect the dissected material because the areas were too big to be catapulted by laser pressure. Collected material was carefully placed in a tube and stored on dry ice/-80°C until RNA isolation with phenol-chloroform extraction method.

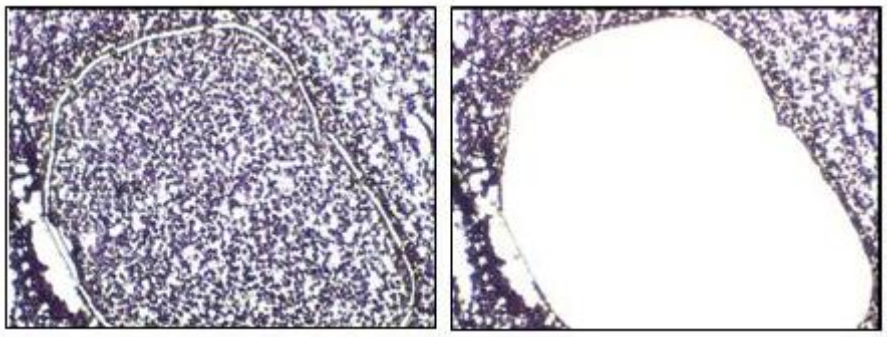


Figure 4.2
Dense Hemalaun staining of the mantel zone served as a border for the laser.

4.2.14 Animals

Animals with C57BL/6 background were used in the study. *P75^{NTR}* (*Ngfr)* exon IV knockout (von Schack et al., 2001) and wild type mice were imported from MRC Toxicology Unit, University of Leicester, United Kingdom. All mice were maintained in the conventional and SPF animal facilities of Max-Planck-Institute of Neurobiology, Martinsried, Germany. Mice will be noted as wild type (WT) and knockout (KO) for wild type and *p75^{NTR}* exon IV knockout mice respectively

Table 4.1 List of mice used for experiments

Wild type mice

Sex	ID. Number	Ear marking	Birth date
M	10.1.2	LEM	07.04.06
M	10.1.4	2REM	07.04.06
F	10.1.5	REM	07.04.06
F	10.1.6	LEM	07.04.06
F	10.2.9	BEM	03.05.06
F	10.2.10	2REM	03.05.06
F	9.3.10	BEM	20.05.06
F	9.3.10	3R2LEM	20.05.06
F	6.6.3	REM	20.05.06
F	6.6.4	LEM	20.05.06
M	9.3.1	REM	20.05.06
M	9.3.3	1R2LEM	20.05.06
M	9.3.2	LEM	20.05.06
M	10.3.2	2LEM	31.05.06
M	10.3.5	2L1REM	31.05.06
M	11.2.5	2LEM	15.06.06
M	11.2.3	BEM	15.06.06

Knockout mice

Sex	ID. Number	Ear marking	Birth date
F	7.1.7	REM	06.03.06
F	7.1.8	LEM	06.03.06
F	7.1.9	BEM,	06.03.06
M	8.1.1	REM	25.03.06
M	8.1.2	LEM	25.03.06
M	8.1.3	BEM	25.03.06
F	8.1.4	REM	25.03.06
F	8.1.5	LEM	25.03.06
M	7.2.2	LEM	12.05.06
M	7.2.4	2REM	12.05.06
M	7.2.5	2LEM	12.05.06
M	7.3.1	REM	09.06.06
M	7.3.2	LEM	09.06.06
M	7.3.3	BEM	09.06.06
F	7.3.4	REM	09.06.06
F	7.3.5	LEM	09.06.06
F	7.3.6	BEM	09.06.06

(LEM: Left ear marking. REM: Right ear marking. BEM: Both ear marking.)

4.2.16 Genotyping

KO mice were genotyped by conventional PCR method using primers as described by von Schack et al., 2001. Genomic DNA has been extracted from a small piece of tail that was clipped after weaning.

PCR Primers

1. 5'-AAGGGGCCACCAAAGAACGG-3'
2. 5'-TGTTGGAGGATGAATTTAGGG-3'
3. 5'-GATGGATCACAAGGTCTACGC -3'

Gel electrophoresis

10 µl template and 2 µl 6x loading buffer were pipeted to each well of a 1.5% agarose gel. Run was performed for 40 min at 70 V. Expected product sizes are 500 bp and 350 bp for wild type and knockout respectively (Fig. 4.3).

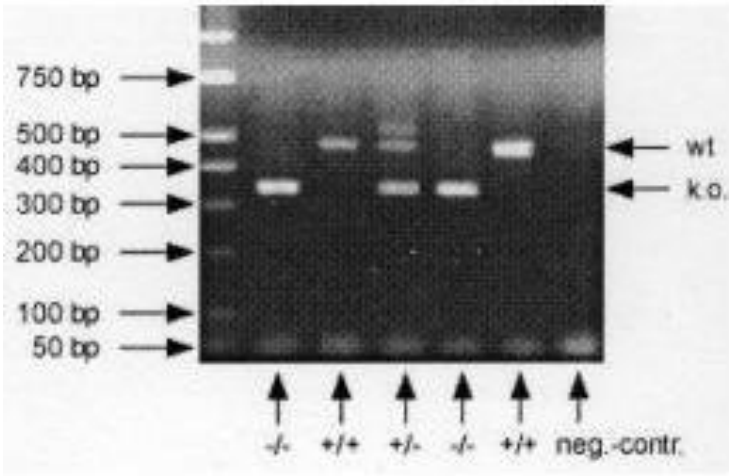


Figure 4.3
Genotyping of *Ngfr* genes by PCR: the 500 bp fragment is derived from the wild type and 350 bp band from the mutant gene. In case of two bands, the mouse is heterozygous.

4.2.17 Immunization

Sheep red blood cells (SRBC)

Sheep red blood cell immunization is a well-established method for the induction of a strong germinal centre response. Purified SRBC (Fiebig Nährstofftechnik, Idstein, Germany) were injected at $5x10^9$ cells/ml in PBS intra-peritoneally (i.p.). A total volume of 200 µl ($1x10^9$ SRBC) was injected to each animal. Non-immunized and 7, 14, 21 day immunized WT and KO mice were used for experiments.

Virus like particles (VLP)

Virus like particles (VLP) of cytomegalovirus (CMV) occur during viral replication in cell culture. VLP lack DNA and only contain fragments of capsid, so they are not infectious. VLP were applied intravenous and 5 μg VLP in 200 μl PBS (25 μg/ml) used for a single immunization. A booster injection was done 60 days later and a concentration of 10 μg/ml has been used. Another group of mice was immunized to monitor the primary Ig response. A VPL concentration of 10 μg/ml was used for this purpose.

To obtain VLPs, human foreskin fibroblasts (HFF) were infected with human CMV and kept in the incubator for approximately 10 days. Later, supernatant was ultracentrifuged (10–40% sucrose density gradient centrifugation at 94.500 g for 60 min, the band material was collected and pelleted by centrifugation at 155.000 g for 60 min); virions, dense bodies and non-infectious particles were separated using a sodium-tartrate-gradient (Talbot and Almeida, 1977). VLPs were kindly provided by Prof. Thomas Winkler (University of Erlangen).

The utilization of VLP for the immunization has two main advantages. Firstly, VLP do not require being co-injected with an adjuvant, leading to a more specific response, and secondly, the availability of capsid glycoprotein-B allows very sensitive ELISA detection of antigen specific antibodies.

Ovalbumin (OVA)

Ovalbumin was emulsified in complete Freund's adjuvant (CFA). The final concentration was 1 μg/ml and the injections were done subcutaneously (s.c.), 100 μl per mice. A booster injection was done 60 days later and a concentration of 1 μg/ml used. For the booster injection incomplete Freund's adjuvant (IFA) was used as adjuvant. Additional to boosting assay, a second group of mice was immunized for a primary Ig response. A concentration of 1 μg/ml OVA+CFA was used for this purpose.

4.2.18 Serum collection

Mice were bled by retro-orbital puncture and blood was collected with a glass capillary. Samples were kept overnight at 4°C. Serum was collected after centrifugation at 4°C / 1 g for 10 min and stored at -20°C until further analysis.

4.2.19 Isolation of splenocytes

Spleens of KO and WT mice were homogenized and passed through 40 μm cell strainers (BD). Cells were centrifuged and red blood cells (RBCs) were eliminated by RBC lysis buffer (Qiagen). After recovery of the isotonicity, isolated splenocytes were counted and prepared for FACS analysis.

4.2.20 Statistical analysis

Data are represented as the mean (or average) of at least three values (actual number of biological samples and repeated experiments are always indicated below each figure).

Error bars represent either standard deviation (Sd) or standard error of the mean (SEM), calculated as following: SEM = Sd / √n. For calculation of statistical significance, Mann-Whitney u-test or Student T-test has been used (paired, two-tailed). *P* values smaller than 0.01 were considered as highly significant and marked with two asterisks (**) and *P* values smaller than 0.05 were considered as significant and marked with one asterisk (*). Statistical calculations were made by Microsoft Excel or GraphPad Prizm v4.0.

5. RESULTS

5.1. Human study

Studies focussing on $p75^{NTR}$ are addressed to central and peripheral nervous systems in most of the cases, where neurotrophins and neurotrophin receptors are expressed constitutively and have their main functions. Very detailed expression profiles for various cell types localized in anatomically distinct areas of CNS and PNS have been done. When scientists investigated other organ systems, tissues or cells, new candidates that can secrete or respond to NTs have emerged. There are many reports about the expression of the NGF family of neurotrophic factors and their receptors in immune cells and organs, dated back to the initial period when the NTs and NTRs were newly identified. Unfortunately, it is hard to deduce a clear expression profile due to inconsistency among publications. The lack of highly specific monoclonal antibodies or advanced detection techniques was the main drawback. Therefore it was necessary to investigate the expression pattern on each immune cell subset, both at the transcript and protein level. Three different primer pairs and three different monoclonal antibodies against human $p75^{NTR}$ were used in this study.

5.1.1 All exons encoding the functional domains of $p75^{NTR}$ are expressed in PBMCs and immune organs

$P75^{NTR}$ protein has three major domains that interact with ligands or adapter proteins. The neurotrophin binding domain, intracellular juxta-membrane domain and the death domain (Fig 5.1A). Two different sets of primers were used to detect coding regions of the transcript for functional domains (Fig 5.1A). Resting and activated immune cell subsets possess all functional domains coded by *NGFR* gene. Similar result was obtained for adenoid and tonsil (Fig 5.1B). S-p75 or any other splice variants were not detected in PBMCs or immune organs. This short isoform is called s-p75 and had only been reported in mouse (von Schack et al., 2001, Ibanez 2002).

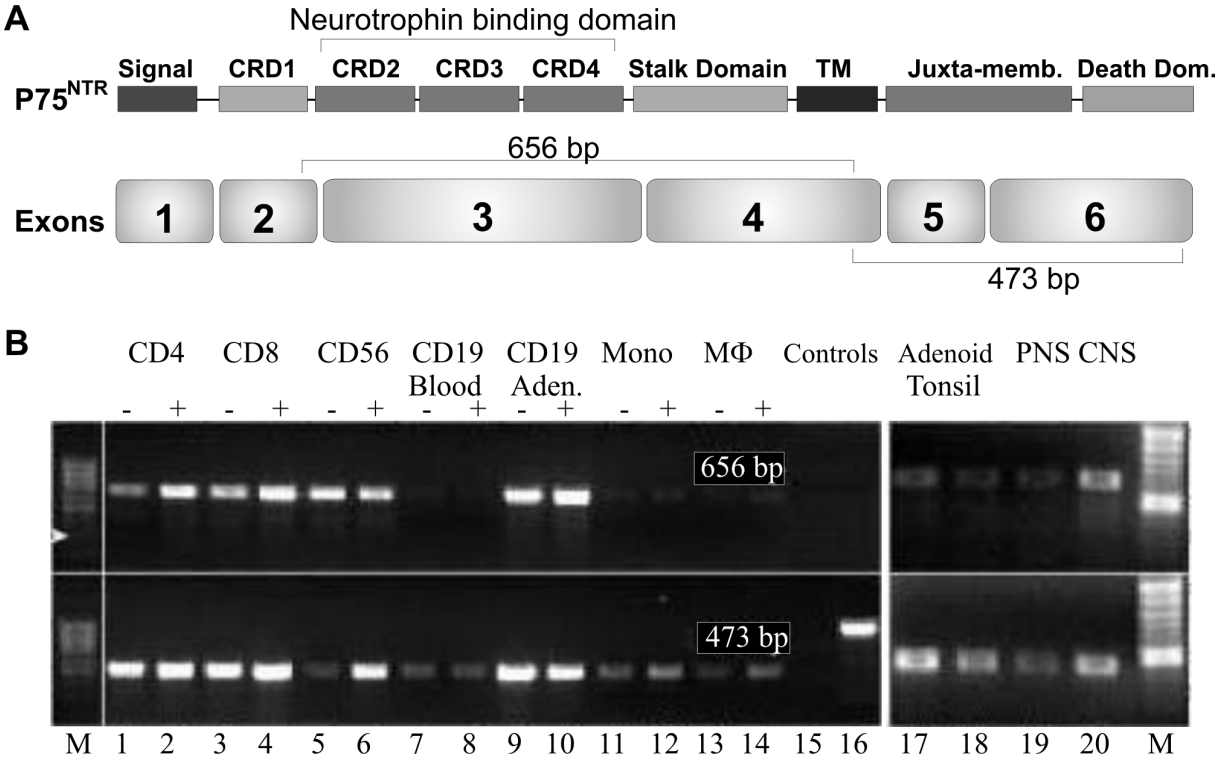


Figure 5.1:

Immune cell subsets and immune organs transcribe full-length p75^{NTR}. *P75^{NTR}* gene transcript consists of 6 exons (A); Exon 1: Signal peptide. Exon 2: Cysteine rich domain 1 (CRD). Exon 3 CRD 2-3-4 also called as neurotrophin binding domain (NTBD). Exon 4: Stalk domain (SD) and trans-membrane domain (TMD). Exon 5: Juxta-membrane domain (JMD). Exon 6: Death domain (DD). Amplicon-1: 656 bp coding NTBD, SD and TMD; Amplicon-2 473 bp coding JM and DD. Various immune cell subsets express *p75^{NTR}* (B) Lanes: 1. CD4$^+$ T cells, 2. CD4$^+$ T cells + PMA-Ionomycin, 3. CD8$^+$ T cells, 4. CD8$^+$ T cells + PMA-ionomycin, 5. CD56$^+$ NK cells, 6. CD56$^+$ NK cells + IL2, 7. CD19$^+$ B cells, 8. CD19$^+$ B cells + pokeweed mitogen (PWM), 9. adenoid CD19$^+$ B cells, 10. adenoid CD19$^+$ B cells + PWM, 11. monocytes, 12. monocytes + LPS, 13. macrophages, 14. macrophages + LPS and immune organs 17. Tonsil and 18. adenoid transcribe full-length p75^{NTR} gene. Peripheral nerve (lane 19) and CNS (lane 20) tissues were used as positive control. Lane 15 is no template control and lane 16 is genomic DNA control. Activation/stimulation period was 24 hour. There was no signal on the 250 bp marked with the white arrow (B, upper panel, left side), concluding that there is no detectable expression of s-p75 in immune cells and organs. Any other immune specific splice variant could not be detected.

5.1.2 Expression of p75 ^{NTR} in PBMCs

In order to better identify the distribution of p75^{NTR} expression on various cell subsets, PBMCs were magnetically separated to obtain highly purified cell populations (with a minimum purity of 91%, data not shown). CD4$^+$ T cells, CD8$^+$ T cells, CD14$^+$ monocytes, CD19$^+$ B cells and CD56$^+$ NK cells (Natural killer) were analysed for p75^{NTR} expression *ex vivo*, and in culture in a resting or activated state. In all cell subsets, p75^{NTR} expression was detectable by PCR analysis (Fig 5.1B). FACS analysis was performed to assess corresponding protein expression levels in each subset (Fig 5.3). In total, 4%, 16% and 20% of

$CD4^+$, $CD8^+$ and $CD56^+$ cells expressed $p75^{NTR}$ respectively, whereas monocytes and B cells did not show any reactivity by FACS (Table 5.1) with two different monoclonal antibodies against $p75^{NTR}$.

Table 5.1

The percentage of $p75^{NTR}$ positive cells in PBMC.

PBMC	$CD4^+$	$CD8^+$	$CD14^+$	$CD19^+$	$CD56^+$
8%	4%	16%	<1%	0%	20%

In unseparated PBMCs, 8% of the cells are $p75^{NTR}$ positive. The main population forming this group are $CD4^+$, $CD8^+$ T cells and $CD56^+$ NK cells. After lineage marker specific cell isolation, these subsets were further analysed individually. 20% of $CD56^+$ NK cells, 16% of $CD8^+$ T cells and 4% of $CD4^+$ T cells were found to express $p75^{NTR}$.

Quantitative PCR analysis showed that *P75^{NTR}* mRNA expression was enhanced upon T cell activation (Fig 5.2). B cells and monocytes only had very low expression and an upregulation was not observed following activation (Fig. 5.2). A weak induction was detected observed NK cells following IL-2 stimulation. Induction of $p75^{NTR}$ in $CD4^+$, $CD8^+$ and $CD56^+$ cells were confirmed by flow cytometry (Fig. 5.3).

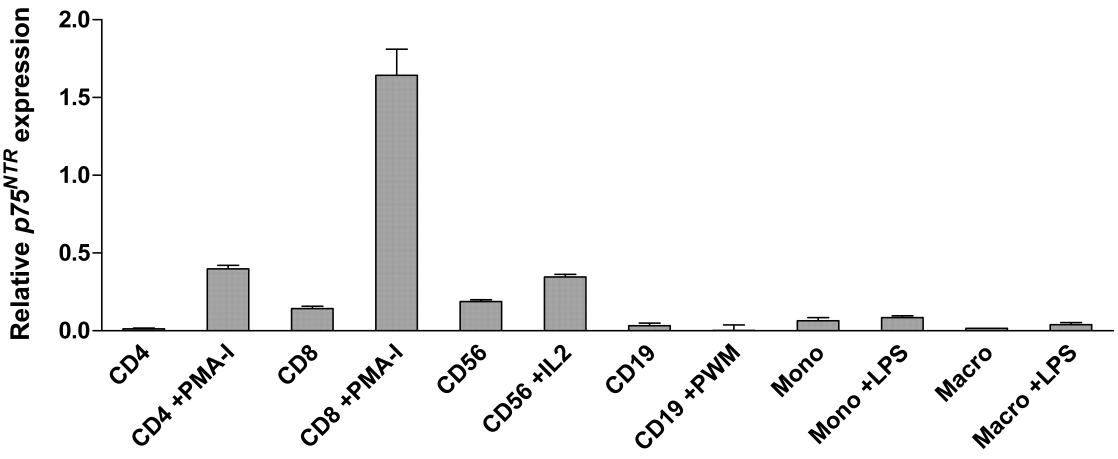


Figure 5.2

Expression of *p75^{NTR}* in immune cell populations has been analysed by TaqMan PCR. In peripheral blood $CD8^+$ T cells and $CD56^+$ NK cells are the main cell populations expressing *p75^{NTR}*. PMA-Ionomycin activation (24 h) of $CD8^+$ and $CD4^+$ T cells resulted in induced expression of *p75^{NTR}*. With IL-2 stimulation (24 h), just a slight induction of *p75^{NTR}* expression in $CD56^+$ NK cells were observed. Monocytes and macrophages had a low basal expression, which does not change upon activation (24 h) with LPS or IFNγ (data not shown). One representative result out of 2-7 independent experiments is shown. Columns represent *p75^{NTR}* relative mRNA expression to reference gene (*Cyclophilin A*). Error bars indicate combined standard deviation of reference gene and *p75^{NTR}*, calculated using replicates.

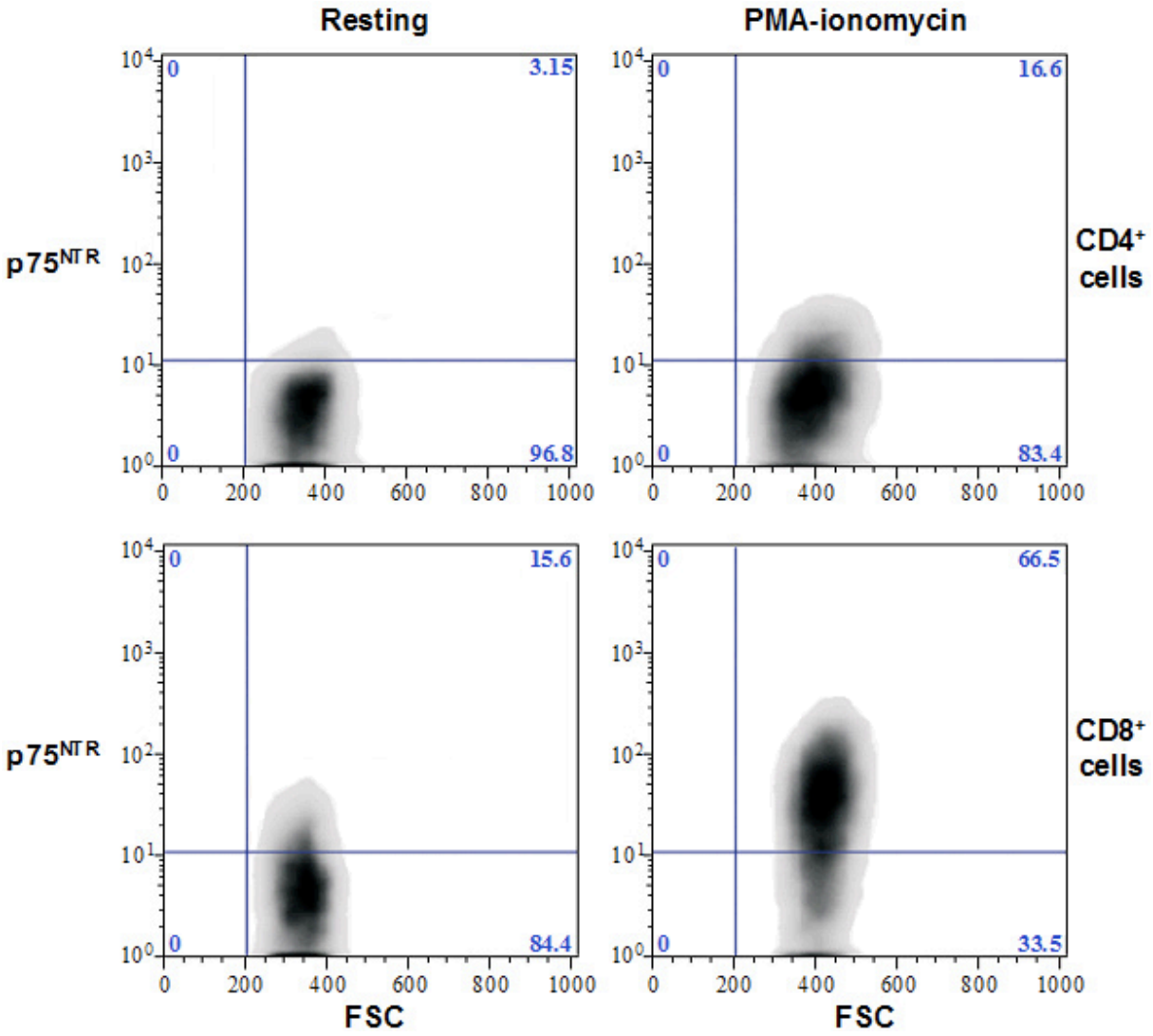


Figure 5.3

P75^{NTR} expression in T cells is induced upon activation. FACS analysis of magnetically separated cell populations: After activation of CD4^{+} and CD8^{+} T cells with PMA+Ionomycin for 24 h, p75^{NTR} expression is induced on both T cell populations, especially on CD8^{+} T cells. Values in the inner corners indicate percentage of cells in respective quadrant. One representative result out of 3 independent experiments is shown. Values located on the corner of each quadrant represent percentage of cells in respective area. Representative result out of 4 independent experiments.

5.1.3 *P75^{NTR}* mRNA levels of immune organs vs CNS tissue

After mapping the p75^{NTR} expression of human PBMC subsets, mRNA levels of immune organs were compared to CNS tissue. Various regions of human CNS (grey matter, white matter, cerebellum and cortex) were used to extract RNA and prepare cDNA. Adenoid, tonsil and spleen samples were analysed and they had similar mRNA level compared to CNS tissue (Fig 5.4). The lowest amount of transcript was observed in PBMC.

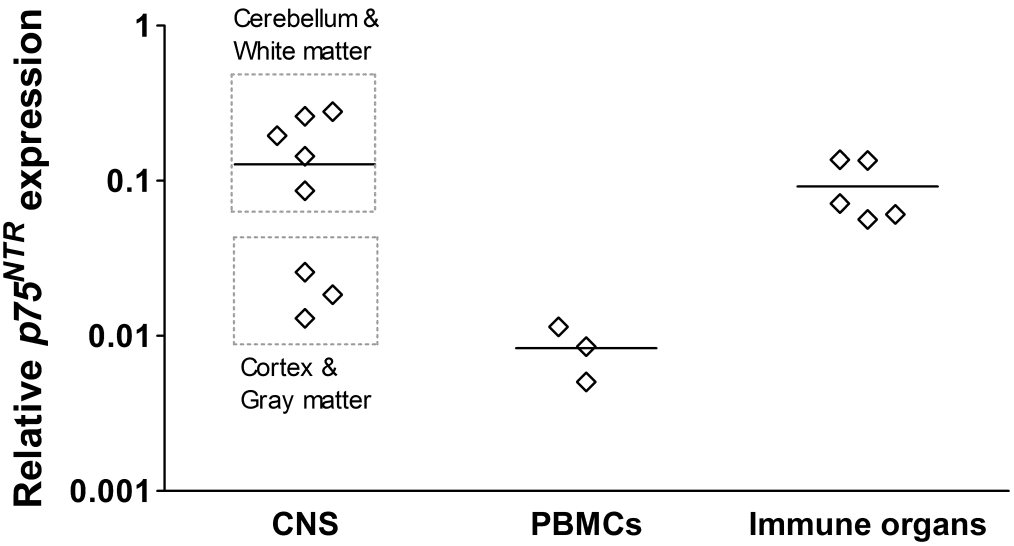
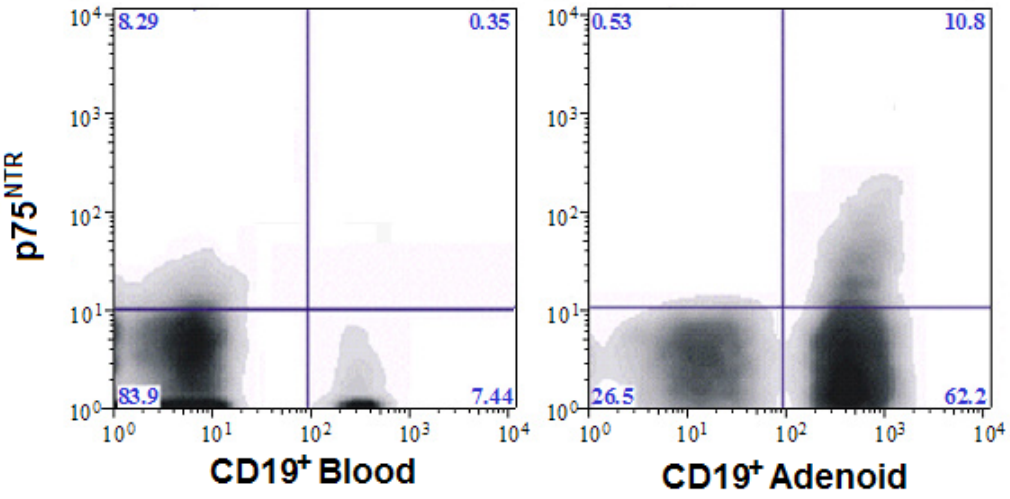


Figure 5.4
P75^{NTR} mRNA levels in CNS and immune organs were compared by TaqMan PCR. CNS and immune organs expressed similar levels of *p75*^{NTR}. Data points of CNS represent different sample preparations from cortex (n=3), white matter (n=3), grey matter (n=3) and cerebellum (n=2). All CNS tissue were obtained from 3 individuals. Ananlyzed immune organs are spleen (n=1) adenoid (n=2) and tonsil (n=2). PBMCs were obtained from blood of healthy donors (n=3). *PPIA* (Cyclophilin A) was used as reference gene.

5.1.4 Expression of p75 ^{NTR} on adenoid B cells

In order to identify the source of $p75^{NTR}$ expression in secondary lymphoid organs, a series of experiments were conducted. First, mononuclear cells (MNC) from fresh human adenoids were isolated by density gradient method and analysed by FACS. About 11% of total adenoid MNCs were positive for $p75^{NTR}$. Almost all $p75^{NTR}$ positive cells were identified as B cells (Fig. 5.5). Among other mononuclear cells, a detectable signal was obtained only from $CD8^+$ cells (1% positive; data not shown).

A



B

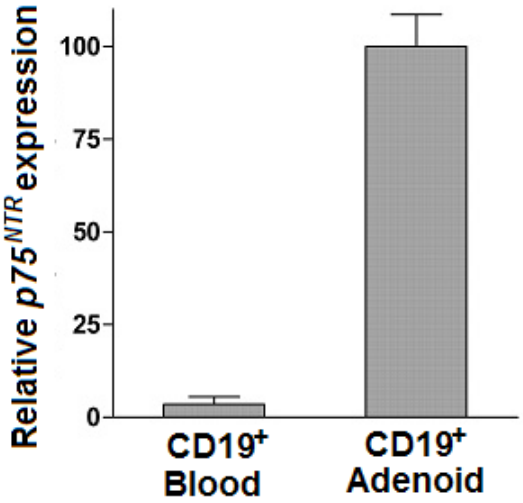


Figure 5.5
Peripheral blood $CD19^+$B cells do not express $p75^{NTR}$, but a subset of adenoid B cells does (A). 10% of unseparated adenoid cells express $p75^{NTR}$. Quantitative PCR analysis of purified blood and adenoid B cells showed similar results (B), n=3 for both groups. Represantative FACS figures from one experiment out of 3. Error bars represent combined standart deviation of reference gene *PPIA (*Cyclophilin A) and $p75^{NTR}$.

In order to classify $p75^{NTR}$ expressing adenoid B cells, further surface staining of purified $CD19^+$ cells using antibodies directed against well-defined B cell markers were performed (Fig. 5.6).

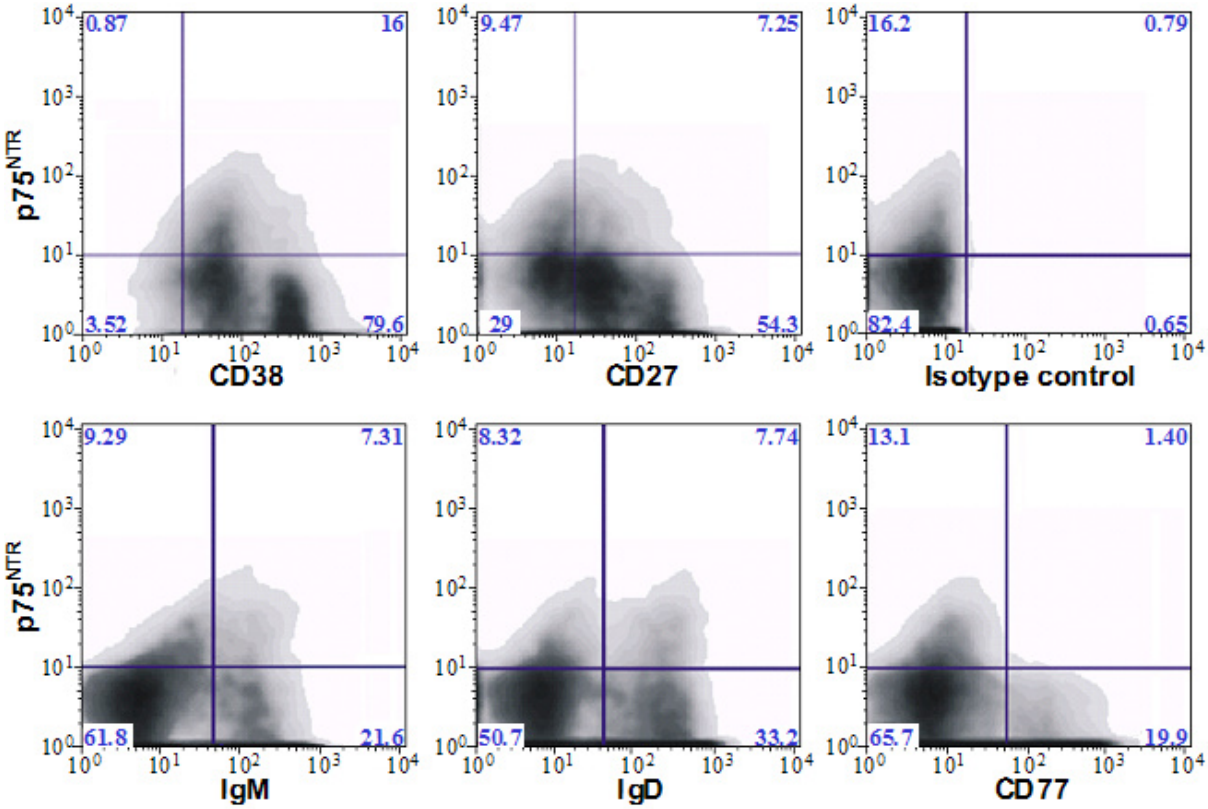


Figure 5.6
$CD19^+$ B cells were isolated from fresh adenoids and analysed by flow cytometry for the expression of $p75^{NTR}$. About 16% of magnetically purified $CD19^+$ adenoid B cells express $p75^{NTR}$. In order to better classify $p75^{NTR+}$ cells, IgM^+, IgD^+, $CD27^+$ (memory B cells), $CD38^+$ (germinal centre B cells) and $CD77^+$ (centroblasts) cells were analysed. The majority of $p75^{NTR+}$ cells express CD38 and not CD77. About 50% of $p75^{NTR+}$ cells are also positive for IgM, IgD or CD27. Mouse IgG1 isotype control used to determine quadrant positions for CD38 and CD27, mouse IgG2a isotype control (not shown) used for IgM and IgD, mouse IgM isotype control (not shown) used for CD77. A representative result from 3 independent experiments is presented. Values on the corner of each quadrant represent percent of cells in each quadrant.

Membrane proteins representing different maturation stages of the adenoid B cells were targeted in parallel with $p75^{NTR}$. $P75^{NTR}$ was distributed on memory B cells ($CD27^+$), germinal centre B cells ($CD38^+$), IgM^+ B cells and IgD^+ B cells. On the other hand plasma cells ($CD138^+$) (data not shown) and centrocytes ($CD77^+$) were negative. With the selection of B cell markers that were used, it was not possible to identify a unique $p75^{NTR}$ expressing population. From the expression pattern, we deduce that $p75^{NTR}$ is present on a subset of

GC B cells and since $CD77^+$ centroblasts are negative, the receptor is present during later stages of maturation.

5.1.5 $p75^{NTR}$ expression in lymphoid organs

In order to map the distribution of $p75^{NTR}$ expression in human immune organs, we used frozen and formalin fixed paraffin embedded (FFPE) material and two different mAbs. Both mAbs against $p75^{NTR}$ showed a very similar staining pattern of germinal centres in tonsils and adenoids (Fig 5.7). In parallel with our flow cytometry results, we could not observe specific $p75^{NTR}$ staining in the T cell area.

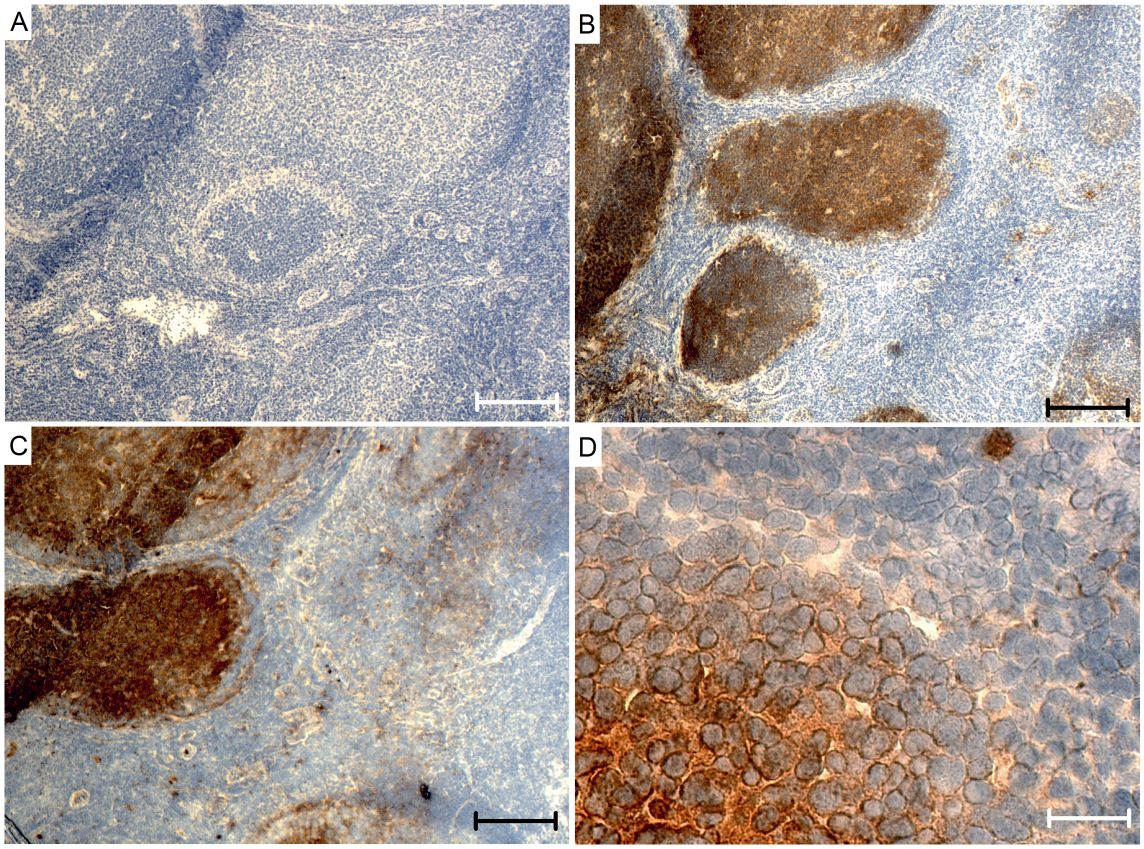


Figure 5.7
Immunohistological staining of tonsil cryosections were performed with two different mAbs against $p75^{NTR}$. Mouse PAP system (Dako) for signal enhancement, hemalaun counterstaining and DAB (Di-amino benzidine - Sigma-Aldrich) substrate were used for visualization. Mouse IgG1 isotype did not show any sign of unspecific staining (A). MAb C40-1457 (BD biosciences) was used in B and mAb ME 20.4 (Sigma-Aldrich) in C and D. Scale bars represent 200 μm in A, B, C and 25 μm in D.

Formalin fixed and paraffin embedded (FFPE) material is more instructive when visualized because tissue integrity is better preserved. Apart from lymph

follicles, basal layer of the squamus epithelium stained positively for $p75^{NTR}$. Additionally, an immunostaining for $p75^{NTR}$ of spleen cryosections was performed. A relatively weak signal was detected in follicles, again on some lymphocytes and FDC like structures, additionally, central arterioles, other blood vessels and splenic macrophages were positive for $p75^{NTR}$ (Fig 5.8).

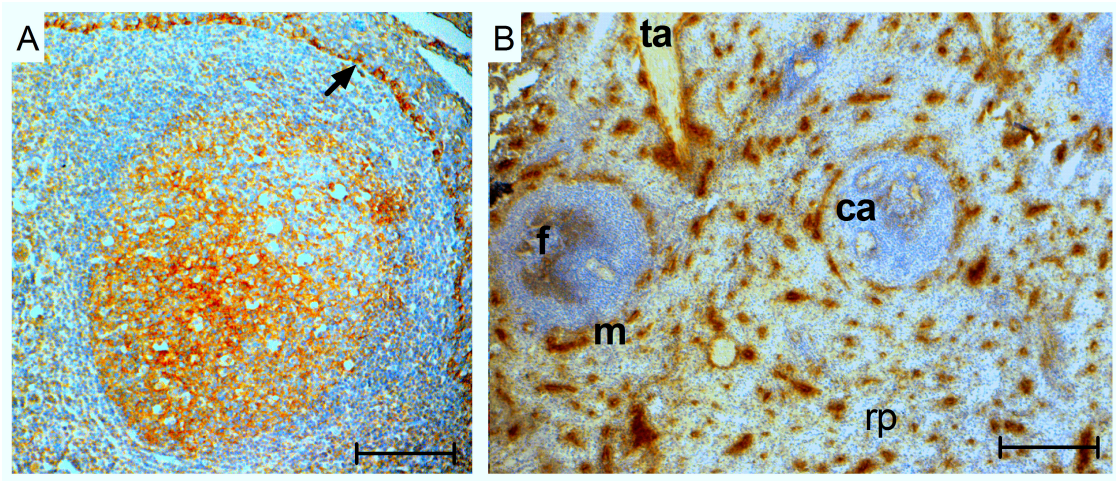


Figure 5.8
FFPE adenoid tissue (A) and frozen sections of human spleen (B) were probed with a mAb against $p75^{NTR}$ (C40-1457). In FFPE adenoid sections additional to the strong follicle staining, the basal layer of the squamus epithelium stained positively as shown with black arrow. Immunohistological analysis of human spleen showed a different pattern of $p75^{NTR}$. Marginal zone macrophages (m) surrounding the white pulp of splenic nodule, central arterioles in splenic nodule (ca), trabecullar artery (ta) stained strongly and cells in the red pulp (rp), presumably macrophages and dendritic cells were also positive. Follicular signal (f) in the white pulp was rather weak compared to adenoid. Scale bar: 200 μm.

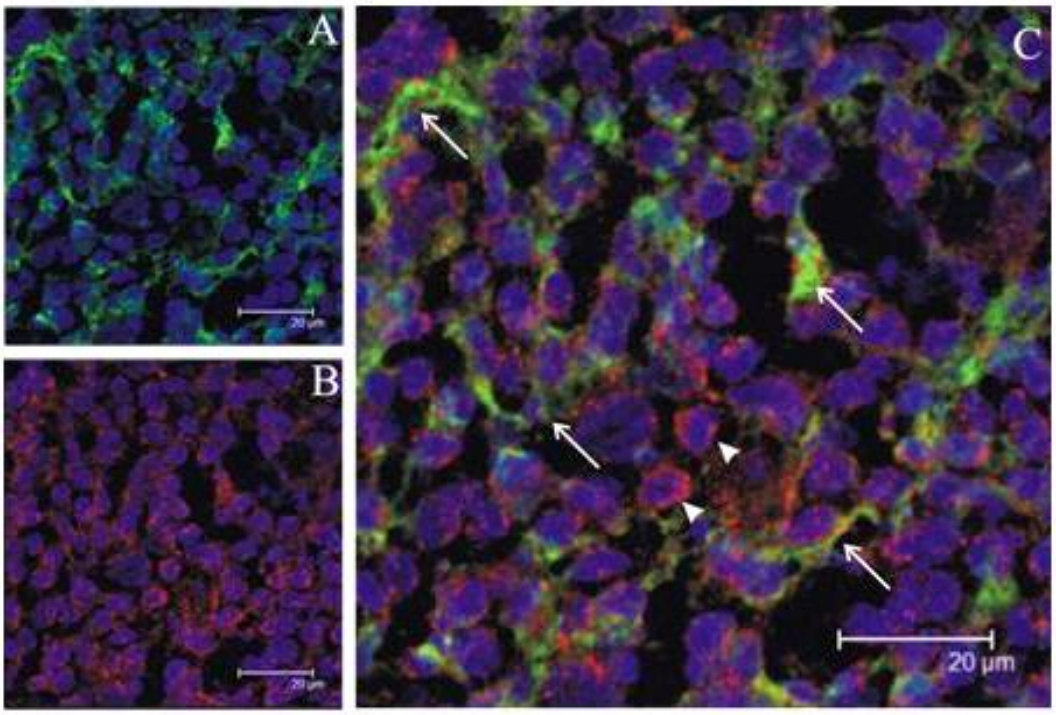


Figure 5.9
Double immunofluorescence assays were performed in human adenoid tissue. A mAb to CD21 (clone 1F8) was used as an FDC marker (A) in combination with $p75^{NTR}$ antibody (C40-1457) (B) and DAPI nuclear staining. In the overlaid picture (C) double positive FDC like structures (arrows) and single $p75^{NTR}$ stained lymphocytes (arrow heads) were present.

For the determination of $p75^{NTR}$ expression on different cell types, double immunofluorescence technique was used. Thereby $p75^{NTR}$ could be localized to FDC. Additionally, single $p75^{NTR}$ positive cells that were negative for the FDC marker were also detected in germinal centres. As a conclusion, FDC and some lymphocytes were identified as $p75^{NTR}$ expressing components of lymph follicles (Fig 5.9).

5.1.6 Expression of BDNF in lymphoid follicles

Looking for ligands of $p75^{NTR}$, we identified BDNF in lymphoid follicles. Double labeling showed that BDNF co-localizes to both B cells ($CD20^{+}$) and FDC ($CD21^{+}$) (Fig. 5.10).

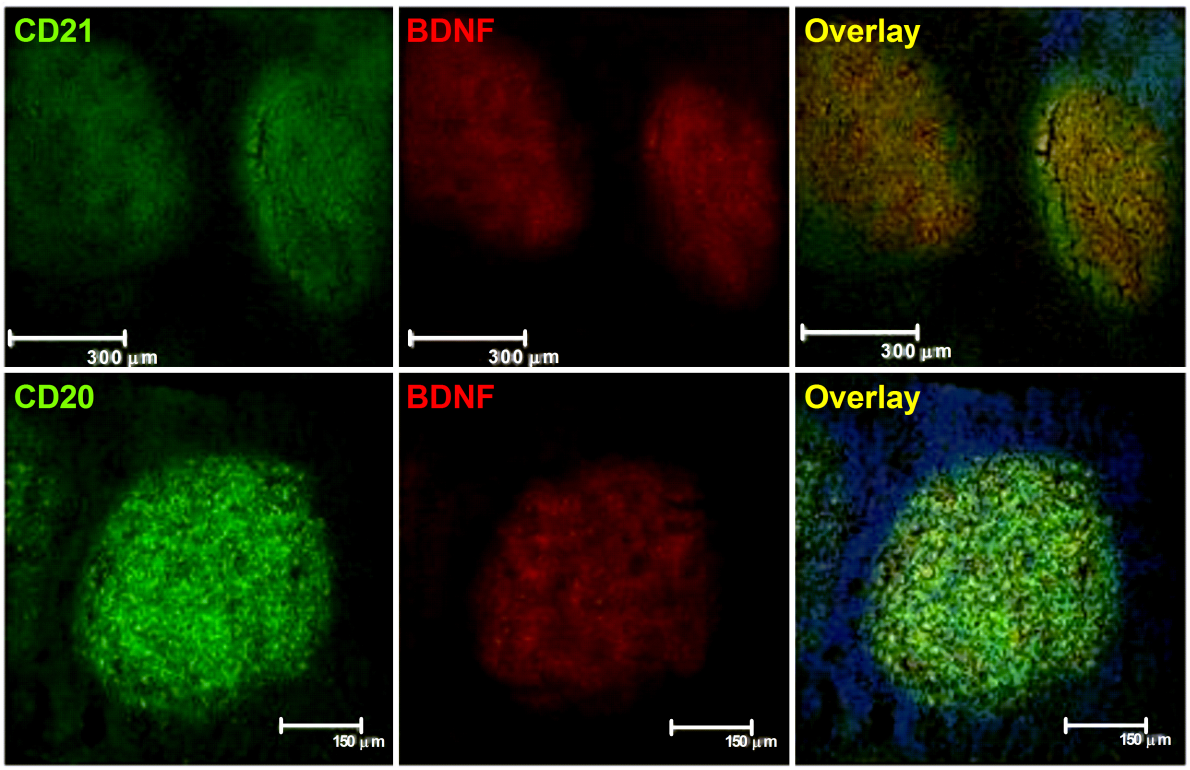


Figure 5.10
BDNF is present in lymphoid follicles. Adenoid cryo-sections were probed with the BDNF specific mAb antibody (clone 35928), in parallel with anti-CD21 (clone 1F8) and anti-CD20 (clone L27) mAbs as markers for B cells and FDC respectively. A clear staining of lymphoid follicles has been observed. Double immuno-fluorescence stainings confirmed co-expression of BDNF with CD21 and CD20.

Since TrkB is considered as the high affinity receptor for BDNF, immuno-staining using antibodies against TrkB receptor were also performed. The expression was detected more prominently outside of the follicular area, in the T cell zone (Fig. 5.11), additionally some macrophages and a weak staining on FDC like structures and lymphocytes. The expression pattern of $p75^{NTR}$ and TrkB is the opposite: $p75^{NTR}$ is the prominent NT receptor in the lymphoid follicles (B cells and FDC) while TrkB seems to be responsible for BDNF signaling outside of the follicles (T cells).

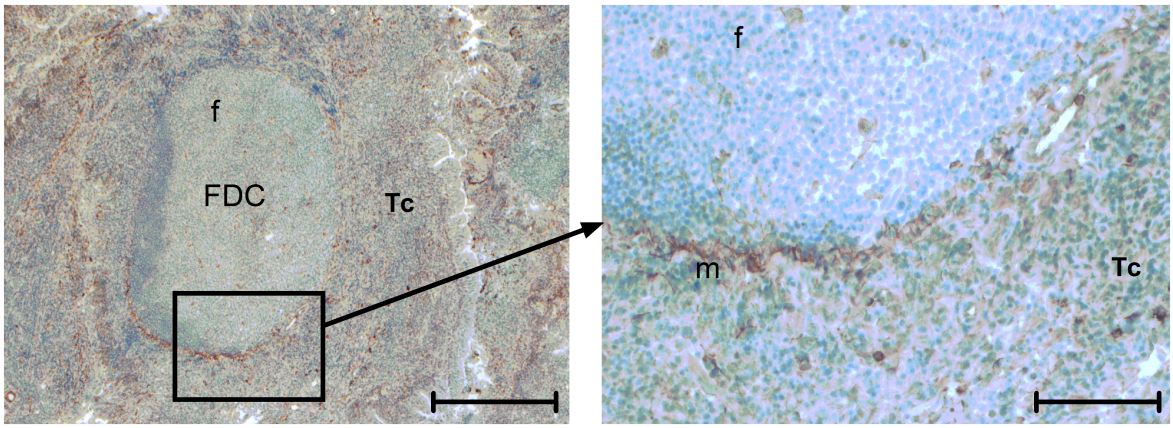


Figure 5.11
TrkB expression (brown staining) in human adenoid is more prominent in the T cell area (Tc), outside of lymphoid follicle (f) (Monoclonal Anti-human TrkB, clone 72509). Weak expression within the follicular area (f) originates from FDCs, lymphocytes and marginal zone macrophages (m). 50x magnification, scale bar: 200 μm on the left and 200x magnification, scale bar: 50 μm on the right. Hemalaun used as nuclear staining.

In order to assess the distribution of *BDNF*, *NGFR* and *NTRK2* mRNA expression in adenoids, frozen sections of adenoids were used for laser micro-dissection. In order to protect RNA integrity, only a fast nuclear staining was done by hemalaun to visualize follicles. Dense nuclear staining of the B cell population of the mantel zone provided a guideline for the laser beam ([1]). Similar cutting patterns were repeated to collect material from T cell area. After RNA extraction from the dissected material, cDNA synthesis and finally for quantitative PCR were performed. Obtained results were in accordance with immunostaining. *BDNF* and *NGFR* transcripts were more abundant in follicles than in T cell area and vice versa for *NTRK2* (Fig. 5.12).

[1] Refer to figure 4.3 in Materials and Methods section for additional details about laser microdissection.

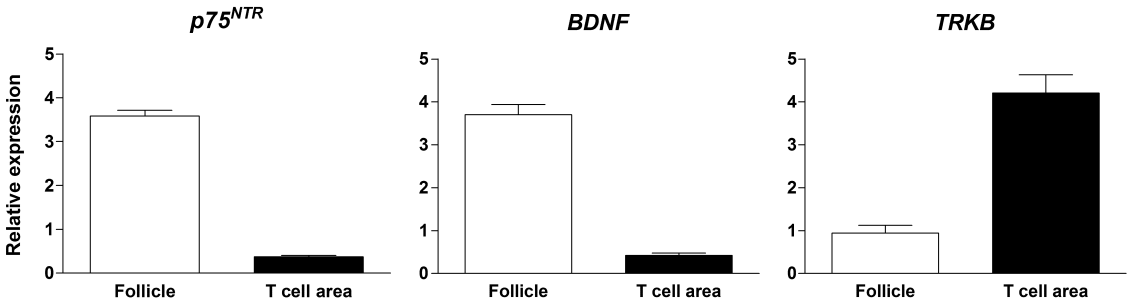


Figure 5.12
The mRNA analysis of laser microdissected lymphoid follicles. 10 μm thick frozen human adenoid sections were placed on PET slides and dried under RNase free conditions. A fast hemalaun staining was performed to visualize lymphoid follicles due to high B cell intensity in the mantel zone. 20 tissue pieces obtained from T cell area and 20 lymphoid follicles were pooled from three different adenoid samples. Collected material was used for RNA isolation, reverse transcription and quantitative TaqMan PCR. Error bars represent combined standart deviation of reference gene (*GAPDH*) and gene of interest.

5.1.7 P75^{NTR} is present in extranodal follicles of Hashimoto's Thyroiditis

It is well known that extranodal follicles develop during several autoimmune diseases like rheumatoid arthritis (RA) or Hashimoto's thyroiditis (HT) (Fig. 5.13). In HT, the immune system attacks the thyroid cells and various auto-antibodies against thyroid peroxidase, thyroglobulin and thyrotropin receptors may be present in the patient's sera. Prolonged autoimmune attack to the thyroid usually causes hypothyroidism. One of the major characteristics of the disease is the occurrence of extranodal lymph follicles in the thyroid tissue (Fig. 5.13).

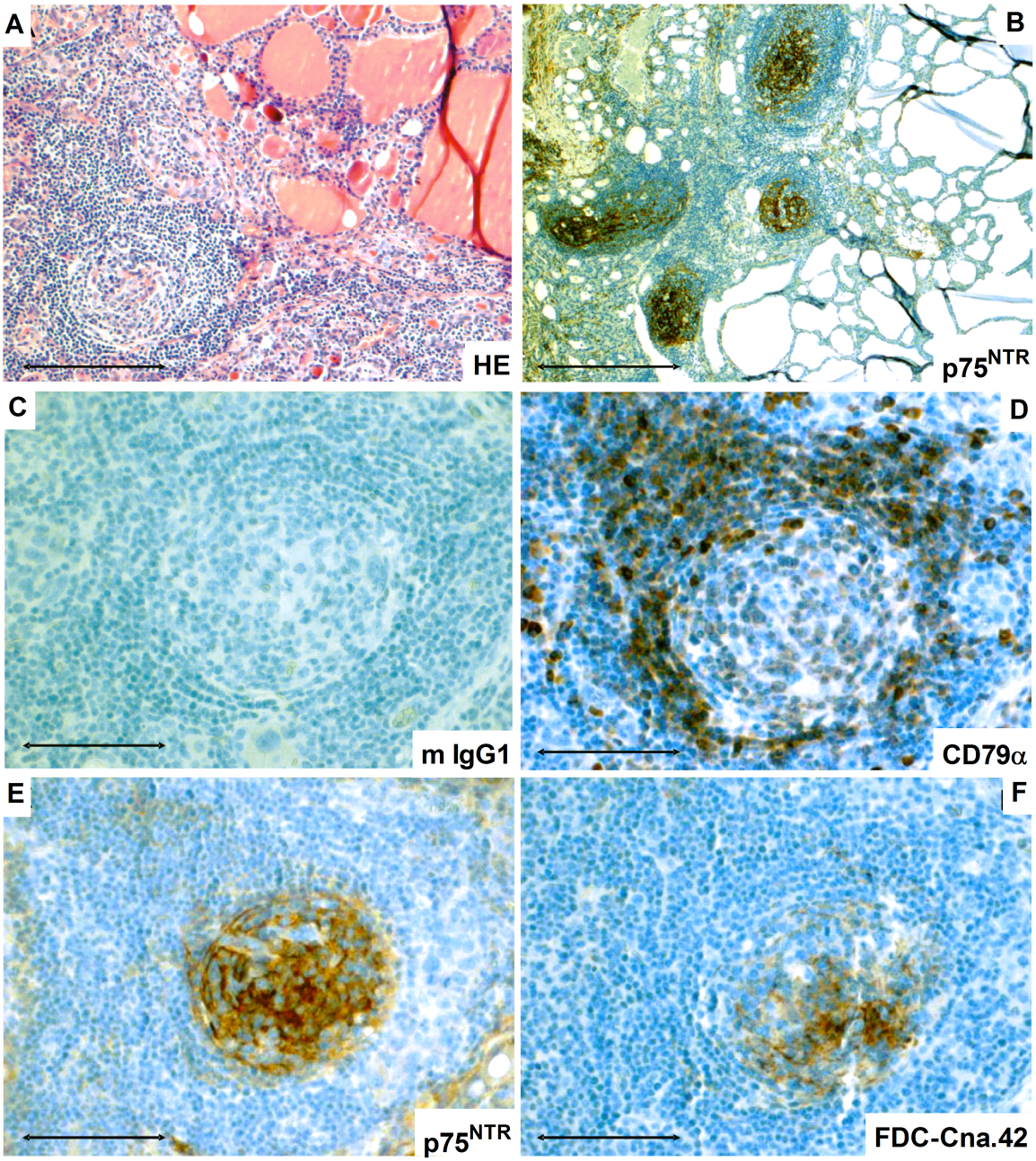


Figure 5.13
Several extranodal follicles within the thyroid in Hashimoto's Thyroiditis. Normal histological pattern of thyroid structure is distinguishable on the upper right area in hematoxylin eosin (HE) stained tissue section (A). Dense lymphocyte population infiltrated the tissue. Follicular dendritic cells in extranodal lymph follicles were stained by $p75^{NTR}$ antibody (C40-1457) (B). $P75^{NTR}$ antibody does not stain B cells in paraffin embedded material. (50x magnification) Serial sections of extranodal follicles were stained with $P75^{NTR}$, FDC (CNA.42) and CD79α (CJB117) specific mAbs. Mouse IgG1 isotype Ab did not cause any background signal (C). $P75^{NTR}$ staining (E) was concentrated in follicular region, similar to adenoid lymph follicles. Further immuno-stainings for B cells (D) and FDCs (F) contributed in understanding the morphology of extranodal follicles. Scalebars: A and B 400 μm; C, D, E and F 200 μm.

The expression of $p75^{NTR}$ in extranodal follicles has not been reported before. For this purpose we compared thyroid tissue samples from healthy indiviuals (n=5) and from patients with Hashimoto thyroiditis (n=5). We used both immuno-histochemistry and quantitative PCR to access the expression profile of $p75^{NTR}$ in extranodal follicles. For all experiments formalin fixed and paraffin embedded material was used. Initially, extranodal follicles were identified morphologically and then by applying established FDC and B cell markers. Two different mAbs against $p75^{NTR}$ performed similarly and showed reactivity with FDC like structures (Fig. 5.13).

A quantitative PCR of 5 HT cases and 5 healthy thyroids showed that $p75^{NTR}$ is upregulated in HT (Fig. 5.14).

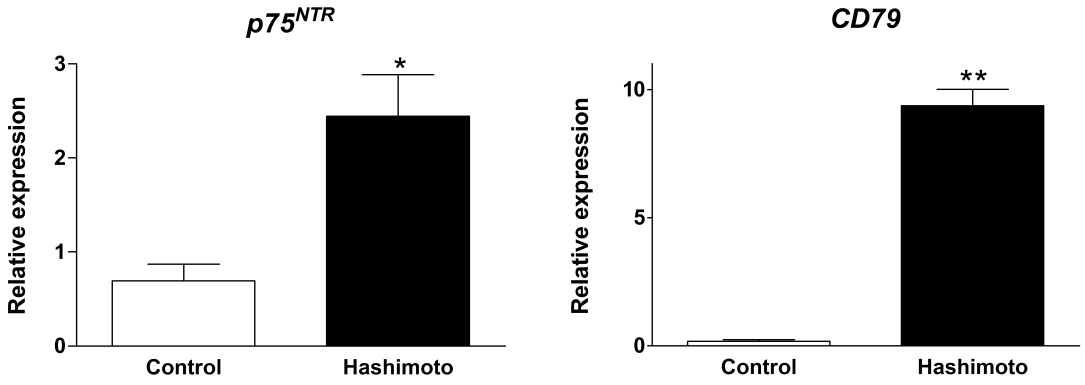


Figure 5.14
The transcript level of *p75^{NTR}* was significantly higher in HT samples as compared to control thyroid ($p<0.05$). *CD79A* was used to mirror B cell presence in the thyroid as a positive control for HT. Scale is individually adjusted for each graph, n=5 for each group. *Cyclophilin A* was used as reference gene. Error bars indicate SEM. (* $p<0.05$, ** $p<0.01$, Mann-Whitney U-test.

5.1.8 $P75^{NTR}$ expression in follicular B cell lymphomas

Another human disease with extranodal follicles is follicular B cell lymphoma. *BCL-2* translocation is the main feature of the follicular B cell lymphoma.

BCL-2 is located in the membrane of the endoplasmic reticulum (ER), nuclear envelope, and in the outer membranes of the mitochondria (Hockenbery et al., 1990). In malignant B cells, the region of chromosome 18 containing the *BCL-2* locus has undergone a reciprocal translocation with the antibody heavy

chain locus of chromosome 14 (Pegoraro et al., 1984). This (14;18) translocation places the *BCL-2* gene close to the heavy chain gene enhancer, which is very active in B cells. As a result, BCL-2 protein is expressed at high levels in these cells, suppressing initiation of the apoptosis process (Williams 1991).

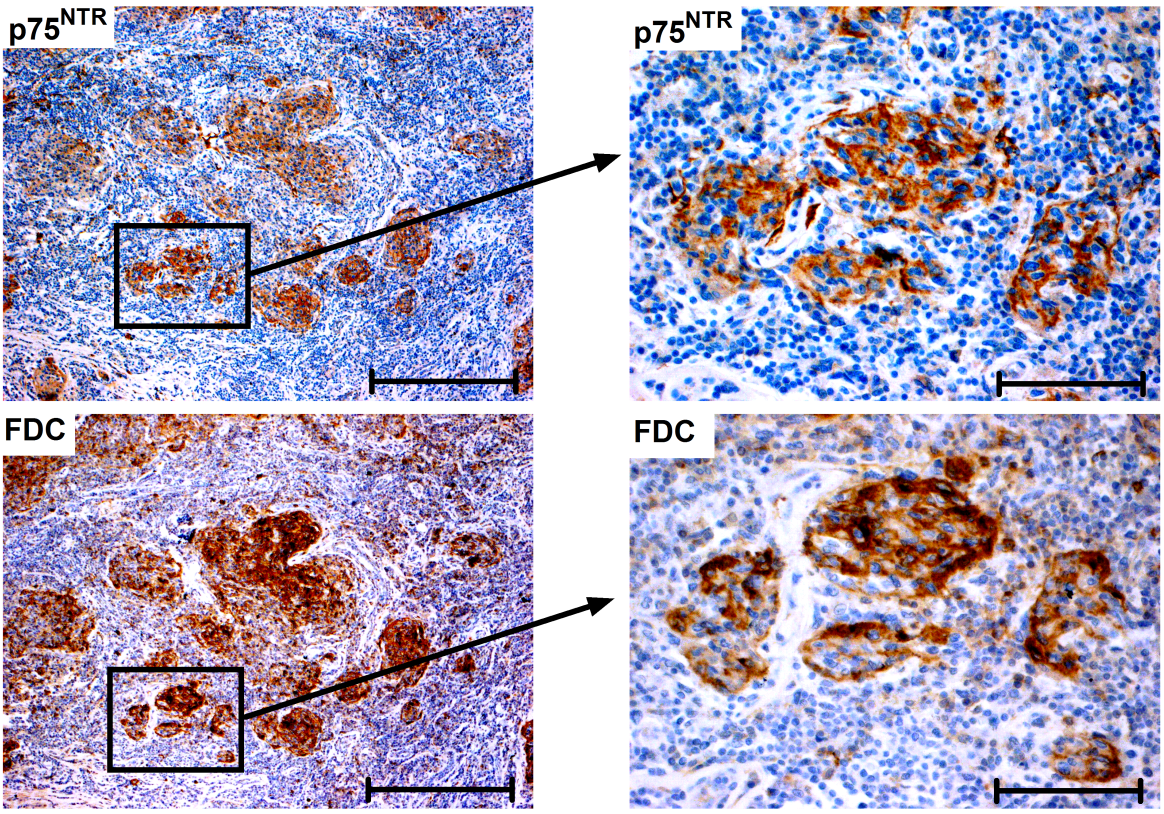


Figure 5.15

Immunohistological analysis of follicular lymphoma cases. Serial sections were prepared from 5 samples of follicular lymphoma cases. The signals from $P75^{NTR}$ (clone C40-1457) and FDC marker (clone CNA42) were co-localized. 200x magnification of the same location had been investigated in order to observe any morphology that might be related to either FDC or other follicular component. In all of the five follicular lymphoma cases, similar observations were recorded. 50x magnification, scale bar: 200 µm on the left and 200x magnification, scale bar: 50 µm on the right. Hemalaun was used for nuclear staining.

5.2. Animal study

5.2.1 P75^{NTR} expression in mouse spleen

In wild type mice, strong expression of p75^{NTR} was observed in the lymphoid follicles (Fig. 5.16) and the expression pattern in the mouse spleen and lymph nodes showed great resemblance to human tonsil/adenoids.

As an initial step, before starting a detailed investigation of the immune phenotype of *Ngfr* exon 4 knockout mice (will be referred as KO or *p75^{NTR}* KO), a series of immunohistological experiments were performed in order to confirm the complete deletion on protein level. As expected, anti-p75^{NTR} antibody did not find any target in the spleen sections of *p75^{NTR}* KO mouse (Fig. 5.16).

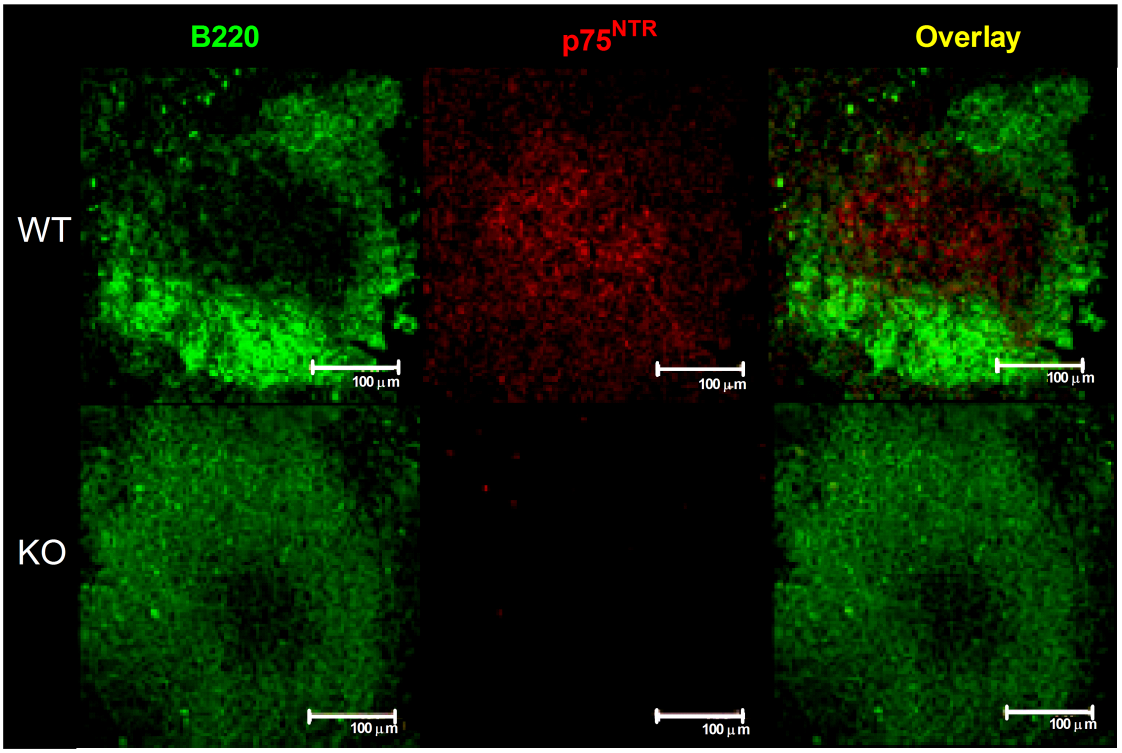


Figure 5.16

Cryosections from spleen of *p75^{NTR}* KO and WT mice were probed with an anti-p75^{NTR} antibody (ab8874) that targets the neurotrophin-binding domain of the receptor. In parallel, B220 antibody (clone RA3-6B2) visualized B cells. WT spleen section showed a lymphoid follicle staining similar to the human adenoid/tonsils (upper panel), whereas KO sections lacked any signal from the anti-p75^{NTR} antibody (lower panel). Absence of p75^{NTR} did not affect formation of follicles.

5.2.2 Morphology and cellular composition of *p75^{NTR}* KO mouse spleen

A wide range of well defined markers that are associated with cellular or morphological compartments were used for comparison of WT and *p75^{NTR}* KO mouse. A notable change in the morphology or the distribution of GC B cells and marginal zone B cells was similar in WT and KO mice. For the identification of different B cell subsets, IgM and IgD markers were used. Laminin antibody served for visualization of morphological structures (basic sinusoidal structure of the spleen, white and red pulp). Moma-1 and FDC-m1 antibodies were used for the identification of marginal zone macrophages and FDCs respectively. CD4 and CD8 marked T cell areas, and finally FITC conjugated peanut agglutinin (PNA) was applied to locate germinal centres (Fig. 5.17)

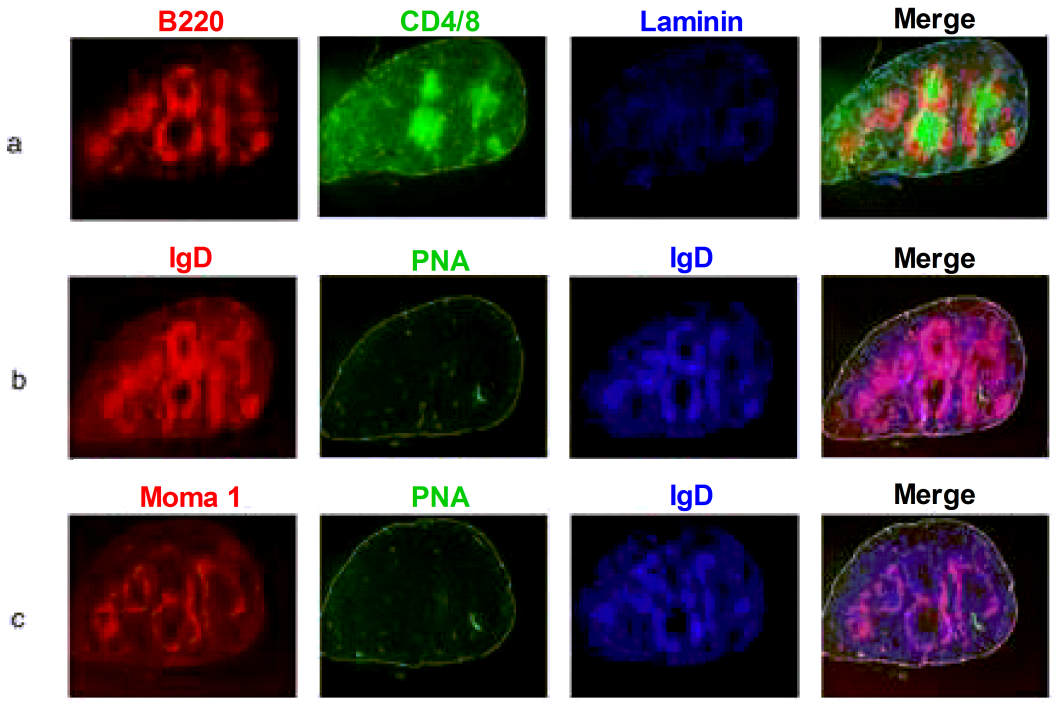


Figure 5.7

Typical immunofluorescence staining of WT spleen in order to visualize cellular compartments. B220 (Red), CD4/8 (green) and laminin (blue) antibodies were used to visualize B and T cell areas and basement membrane components respectively (a), IgD (red), PNA (green) and IgM (blue) markers were included to see the distribution of different B cell subsets and germinal centres (b). Finally, using Moma-1 (red), it is possible to identify marginal zone macrophages and IgD^{+} B cells (blue). PNA (green) shows whether germinal centres exist or not (c).

Spleen sections taken from two sites of the spleen from *p75*^{NTR} KO mouse showed similar morphology compared to WT (Fig. 5.18). Distribution of cellular compartments, the area and the shape of lymphoid follicles appeared to be normal in *p75*^{NTR} KO mice.

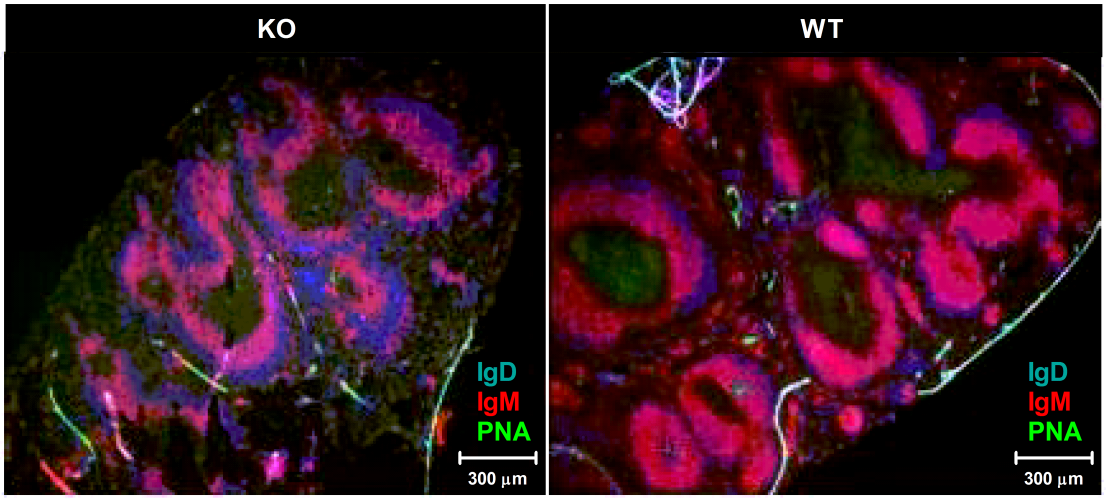


Figure 5.19
Triple immunofluorescence staining of WT (right) and *p75*^{NTR} KO (left) spleen sections were made using IgM (red), IgD (blue) and PNA (green) markers, in order to visualize distinct B cell compartments and germinal centres. There are no obvious morphological differences between *p75*^{NTR} KO and WT spleens. The shape and size of lymphoid follicles are comparable; the organization of distinct cellular compartments seems not to be grossly affected by the deletion of *p75*^{NTR}.

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5.2.3 SRBC immunization of *p75*^{NTR} KO mouse

After the investigation of the WT and KO spleens under resting conditions, sheep red blood cell (SRBC) immunization was performed in order to induce a germinal centre response. Three time points were selected for analysis: 7, 14 and 21 days. For each time point three WT and three KO mice were used. Similar to non-immunized animals, spleens of immunized ones did not show obvious morphological differences (Fig. 5.18). Germinal centre shapes and areas were compared and quantified. PNA was used as a GC marker in combination with anti-IgM and anti-IgD.

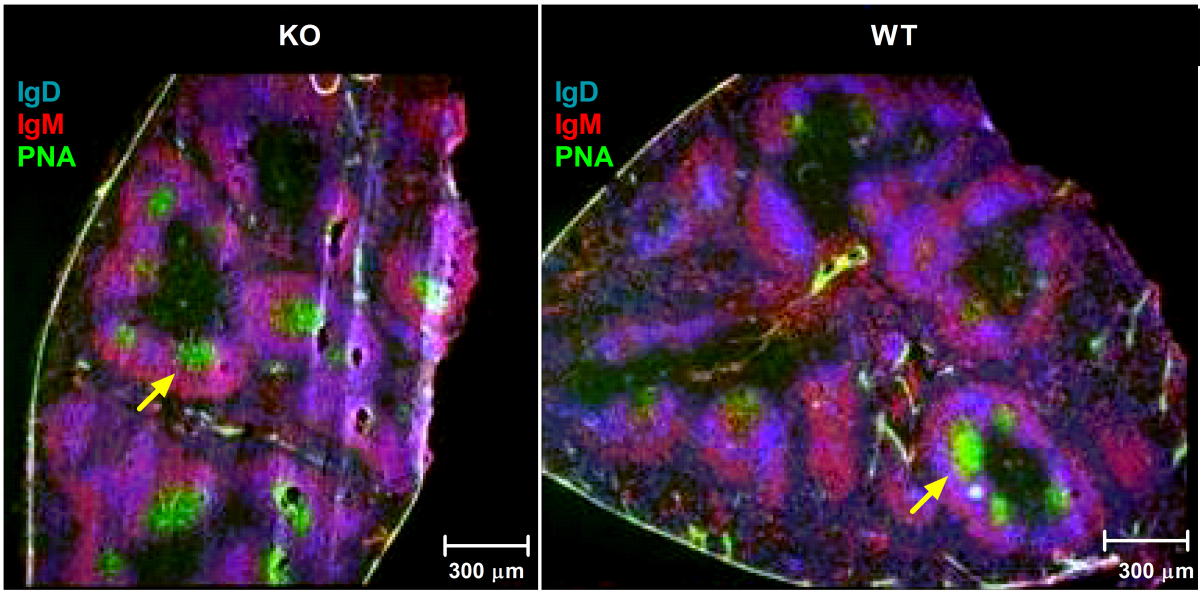

Figure 5.19
Spleen cryosections from non-immunized and 7, 14, 21 day SRBC immunized WT and KO mice were stained with IgM, IgD and PNA markers to pinpoint germinal centres. Representative spleen sections of 7-day SRBC immunized animals are shown. Typically, a dense green PNA signal surrounded by a purple IgM staining shows a germinal centre (arrows). In the KO spleen (left), there are more germinal centres than in the spleen of the WT (right).

For the quantification, sections from three distinct sites of the spleen were analysed for each mouse in a blind manner. In all of the three different time points after the immunization and in nonimmunized animals, *p75*^{NTR} KO mice had more germinal centres per section, compared to wild type (Fig. 5.20).

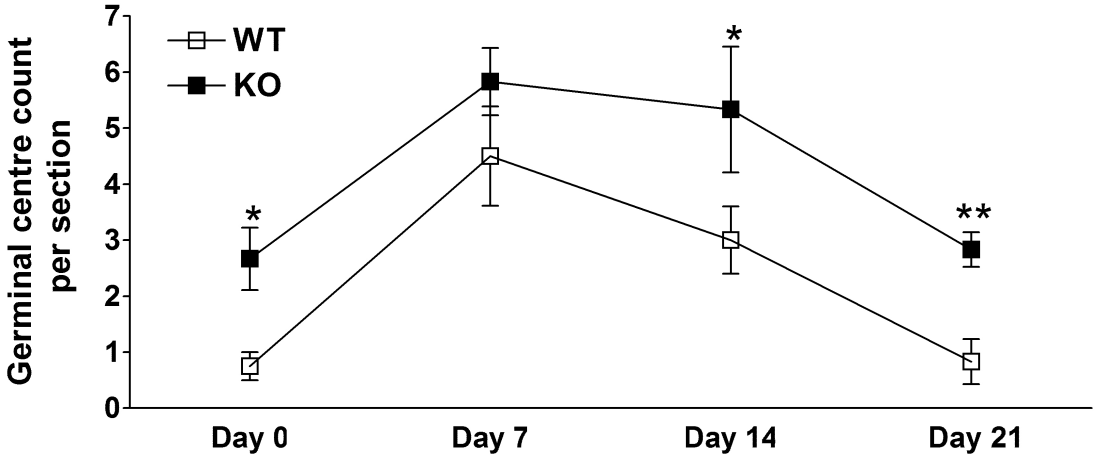

Figure 5.20
Three WT and three *p75*^{NTR} exon 4 KO mice were analysed for each time point. Three anatomically distant sections from each spleen were used for quantification of the GCs (nine sections were used for GC quantification per data point). KO animals have significantly more germinal centres per observed section compared to wild type on day 0,14 and 21 (error bars represent SEM, ∗: p<0,05 and ∗∗: p<0,01).

5.2.4 Cellular composition of *p75^{NTR}* knockout mice

In parallel with the experiments intended to compare the morphology of the spleen, a thorough analysis of splenocytes was performed to compare cellular distribution of the *p75^{NTR}* KO mice compared to WT. A small portion of each spleen was used to isolate MNCs.

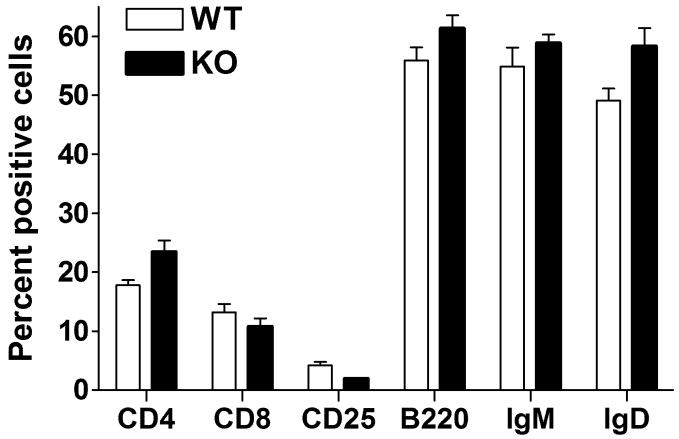

Figure 5.21
A total of three WT and thee KO mice were analysed for possible alterations in splenocyte compositions. There were no significant differences in T and B cell composition. Error bars represent standard deviation.

Splenocytes were probed with a wide range of cell and state specific markers in combinations to allow quantitative comparison of different cell subsets. Differences that were observed between KO and WT animals (Fig. 5.21) were not statistically significant. All three B cell markers (B220, IgM, and IgD) showed the same pattern of expression throughout the immunization period (data not shown).

5.2.5 Antibody production of *p75^{NTR}* KO mice immunized with SRBC, VLP and OVA.

Following the SRBC immunization that was performed to induce a strong GC response, further WT and KO mice were immunized with virus like particles (VLP) and ovalbumin (OVA). After SRBC immunization, blood sampling and serum preparation was done at day 7, 14 and 21. Total and antigen specific IgG and IgM levels were measured by ELISA (Fig. 5.22).

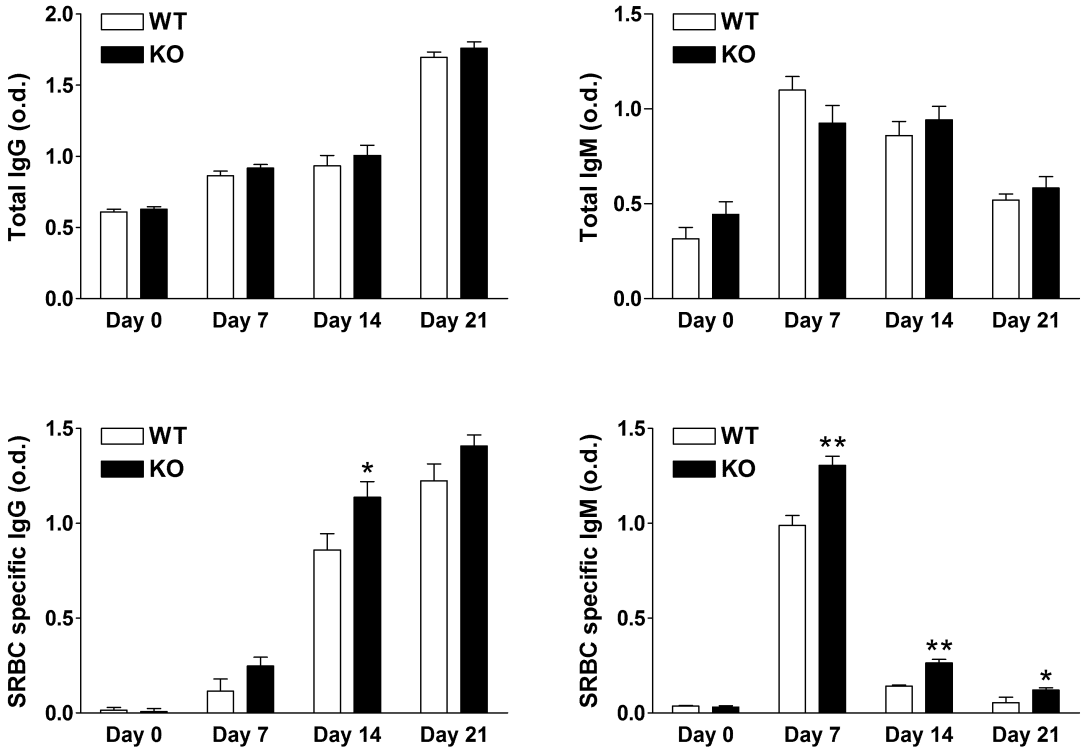

Figure 5.22
Blood sampes were collected on at day 0, 7, 14 and 21 following the SRBC immunization. Serum samples (diluted 1:100 for IgM assay and 1:1000 for IgG assay) were analysed for total and SRBC specific IgG and IgM. There was no difference in total IgG and IgM levels, but SRBC specific IgM levels were higher in KO mice at day 7, 14 and 21 whereas SRBC specific IgG levels were different only on day 14 (o.d.: optical density, error bars represent standard deviation, n=3 for all groups, ∗: p<0,05 and ∗∗: p<0,01).

There was no difference in total IgG and IgM amounts between WT and KO mice. When the amount of SRBC specific IgG and IgM were measured, KO mice had increased levels of SRBC specific IgM for all time points, and additionally SRBC specific IG on day 14 only.

VLP immunization aimed to observe the memory function. Therefore primary Ig response was measured at day 7 and 14 after the initial immunization (Fig. 5.23). VLP specific IgM production was higher in *p75*^{NTR} KO mice. There were no significant differences in VLP speific IgG levels.

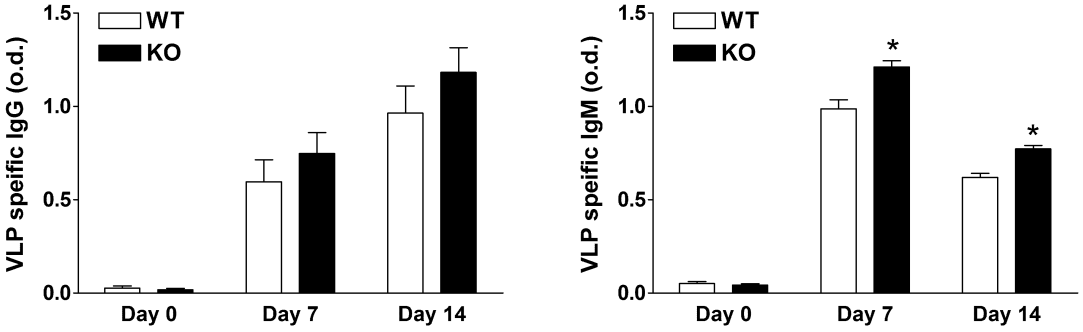

Figure 5.23
For quantifying primary VLP specific Ig production, blood was collected before immunization, as well as 7 and 14 days after immunization. Three KO and three WT mice were analysed for each time point, error bars stand for the standard deviation. The serum was diluted 1:100 for IgM and 1:1000 for IgG assays. VLP specific IgM production on day 7 and 24 is higher in *p75*^{NTR} KO mice whereas there was o diffrence in VLP specific IgG levels (n=3 for each group, ∗: p<0,05).

For the quantification of the Ig response, a booster immunization was performed at day 60. Blood sampling was done before booster and 7 days after booster. First, serum samples were analysed for the total IgG and IgM content. No difference was observed between WT and KO (Fig. 5.24) after the initial and booster immunizations. Quantification of VLP specific Ig yielded a similar result to RBC immunization; VLP specific IgM titer was higher in KO mice during the primary and booster response while VLP specific IgG levels were similar (Fig. 5.23 and 5.24).

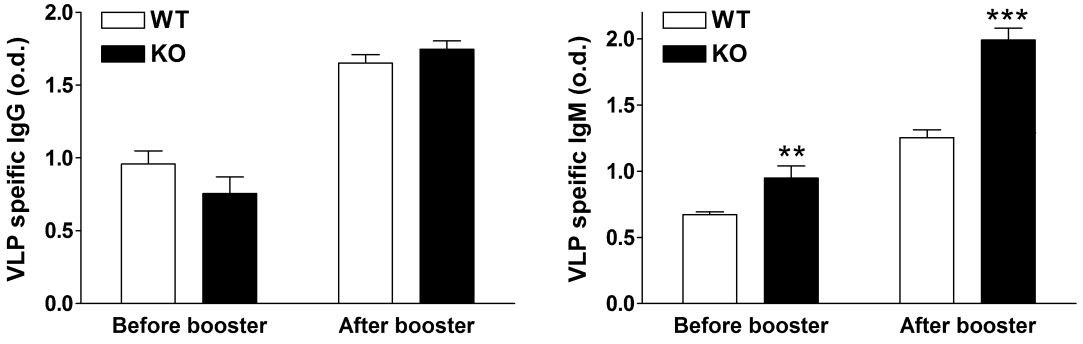

Figure 5.24
Possible alterations in memory response due to the deletion of *p75*^{NTR} were tested by a booster injection at day 60 followed by blood sampling at day 67. Total IgG and IgM production was found to be similar in KO and WT. VLP specific IgM production both before and after the booster immunization was significantly higher in KO mice compared to WT (n=3 for all groups, ∗∗: p<0,01 and ∗∗∗: p<0.001, two tailed t-test, error bars stand for the standard deviation). The serum has been diluted 1:200 for IgM and 1:800 for IgG assays.

Finally, as a third antigen, OVA was injected to induce an immune response. Exactly the same time points for primary and booster immunizations were done as in VLP immunization. There was no difference between WT and KO in terms of total IgG and IgM both for primary (data not shown) and booster responses (Fig. 5.25). On the other hand, just like SRBC and VLP, OVA-specific IgM production was significantly higher in KO mice after initial (day 7) and booster injections (day 67) (Fig. 5.25). OVA specific IgG production was similar in WT and KO (data not shown).

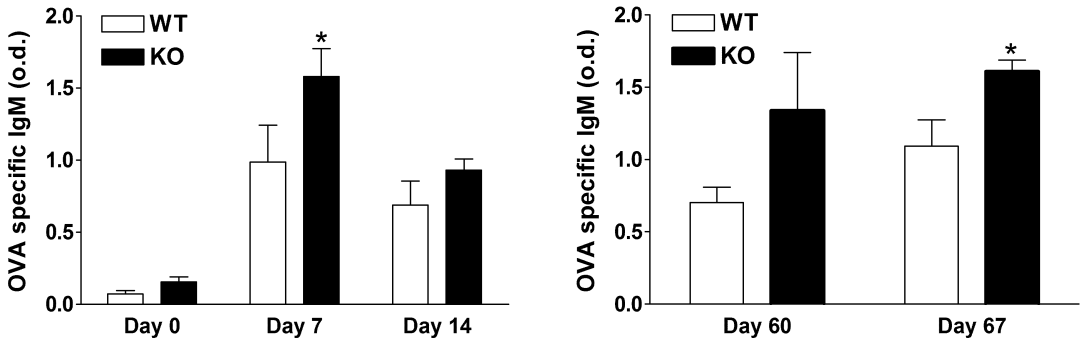

Figure 5.25
OVA immunized mice were compared for primary Ig production; blood was collected before immunization and at day 7 and 14 after immunization. OVA specific IgM production was higher in *p75*^{NTR} KO mice at day 7. Memory response was assessed by a booster injection at day 60 followed by blood sampling at day 67. OVA specific IgM production was significantly higher after booster at day 67 (n=3 for all groups, ∗: p<0,05, two tailed t-test). Serum dilution is 1:200 for day 0, 7 and 14, 1:400 for day 60 and 67.

Experiments performed with all three immunogens yielded parallel result in terms of antigen specific IgM production. Table 5.2 summarizes serum Ig quantification experiments performed by ELISA. Ig classes A and E were also tested and no difference has been observed between KO and WT (data not shown).

Table 5.2: Summary of the primary and secondary IgG and IgM response to the three applied antigens.

Immunization➔	SRBC primary	VLP primary	VLP secondary	OVA primary	OVA secondary
Total IgG	KO = WT	KO = WT	KO = WT	KO = WT	KO = WT
Total IgM	KO = WT	KO = WT	KO = WT	KO = WT	KO = WT
Antigen specific IgG	KO > WT **d14: p<0.05**	KO >= WT	KO = WT	KO = WT	KO = WT
Antigen specific IgM	KO > WT **d7, 14: p<0.01** **d21: p<0.05**	KO > WT **d7, 14: p<0.05**	KO > WT **d60: p<0.01** **d67: p<0.001**	KO > WT **d7: p<0.05**	KO > WT **d67: p<0.05**

Table 5.2
WT and KO mice were immunized with: Sheep red blood cells (SRBC), Ovalbumin (OVA) and virus like particles (VLP). Total and antigen specific IgG and IgM levels for primay response were measured 7, 14 and 21 days after first immunization. Secondary response was addressed 7 day following the secondary immunization done at day 60. Antigen specific IgM quantities were significantly higher in *p75*^{NTR} knockout mice during both primary and secondary response against all three antigens used. Two tailed, paired student t-test was used for statistical analysis, n=3 for all groups.

6. Discussion

The cells that form tissues need to properly communicate with each other for consistency of relevant functions. Although oligosaccharides, lipids, nucleotides or other small molecules act as signaling components, cell-to-cell signaling mainly relies on protein-protein interactions. In most cases, cells do communicate only with neighbouring cells depending on physical contact followed by interaction of surface receptors and membrane bound ligands (juxtacrine signaling). Additionally, cells may secrete factors that immediately act on themselves (autocrine signaling) or on distant cells (paracrine signaling). These soluble proteins have a wide range of roles from homeostasis to hormonal regulations. Neurotrophic factors and their receptors have many roles in this context.

The strategy to investigate ligand receptor interaction is first to locate cells or tissues abundantly expressing those proteins and looking for immediate responses upon blockage or activation of signaling components. Accordingly, dated back to the initial period when the neurotrophins and neurotrophin receptors were newly identified, most of the studies were focussed to central and peripheral nervous system where these proteins have their main functions. Nevertheless, during following years, many studies have shown the expression of NGF family of neurotrophic factors and their receptors in other cells and organs, including several components of the immune system. This study is the first to systematically describe the dynamic expression of $p75^{NTR}$ on various human immune cell subsets.

Ex vivo analysis of human peripheral blood lymphocytes revealed that $CD56^+$ NK cells, $CD8^+$ and $CD4^+$ T cells express $p75^{NTR}$ and the expression level of both transcripts and proteins is induced upon activation. The increased level of $p75^{NTR}$ on human $CD8^+$ T cells remained high up to 48 hours following activation, whereas the expression of $CD4^+$ T cells went back to initial level (Data not shown). Since, T cells were previously reported to produce NGF (Ehrhard et al., 1993b; Santambrogio et al., 1994; Lambiase et al., 1997) and BDNF (Besser and Wang, 1999, Kerschensteiner et al., 1999 and Berzi et al., 2008), it is worthwhile to consider autocrine or paracrine signaling in T cells through $p75^{NTR}$, TRKA and TRKB. Herda et al. reported that CD4, CD8 T cells and NK cells express sortilin receptor and systemic deletion of sortilin resulted in a compromised immune response (Herda et al. 2012). It is possible that $p75^{NTR}$-Sortilin receptor complex and proNGF/proBDNF mediated apoptosis play a

role in T cell functions. Thus, involvement of $p75^{NTR}$ signaling needs to be elaborated under this context.

Following the increase in reports pointing out the presence of neurotrophin receptors and production of neurotrophic factors on different immune cell populations, scientist investigated inflammatory conditions involving the nervous system. Studies on EAE (experimental autoimmune encephalomyelitis) with *Ngfr* exon 4 knockout mice revealed a more severe disease course compared to WT mice (Copray et al., 2004). In parallel, an increased infiltration of T cells into the spinal cord has been reported (Küst et al., 2006). Concerning the T cell infiltration, a similar observation has been made during neuronal regeneration experiments involving peripheral transsection in a mouse model of facial nerve injury. In both *Ngfr* exon 3 and exon 4 mutant mice models, absence of $p75^{NTR}$ led to an increased recruitment of $CD3^+$ T lymphocytes in the axotomized facial motor nucleus (Gschwendtner et al., 2003). In both EAE and facial nerve injury studies, researchers have not considered or discussed any possible outcomes of the absence of $p75^{NTR}$ expression in activated T cells and NK cells, which have the highest potential for infiltration upon injury or inflammation.

It is clear that the ideal strategy to investigate the possible functions of a gene on an immune cell subset would rely on systems that allow selective deletion of that gene on a specific cell subset or lineage. To our knowledge, there is no study that has utilized conditional knockout mice, RNA silencing or inducible gene deletion systems to study functions of $p75^{NTR}$ in the immune system. This was a limitation for researchers but now there is a conditional $p75^{NTR}$ knockout line (Bogenmann, et al., 2011), and it is also possible to engineer different experimental models by using CRISPR/Cas9 system (Huang et al., 2017).

Apart from T cells and NK cells, the expression of $p75^{NTR}$ in peripheral blood B cells is an issue of concern since there are contradicting reports (Brodie et al., 1996, Schenone et al., 1996). In this study, less than 1% of peripheral blood B cells had $p75^{NTR}$ protein, just above the detection limit. On the other hand, a considerable expression of $p75^{NTR}$ was observed in magnetically isolated adenoid B cells. Further investigation of adenoid B cells that express $p75^{NTR}$, revealed sub-populations bearing IgM, IgD, CD38 and CD27 but not CD77. These markers are differentially regulated in secondary lymphoid organs during arrival, selection, elimination, maturation and class-switch phases. Whether all of the B cells in the lymphoid

follicles express $p75^{NTR}$ during a certain phase of their selection/elimination process or the expression is only associated to specialized B cells remains unclear.

The expression of $p75^{NTR}$ in the tonsil and adenoid was not confined only to B cells. When stained with additional surface markers, the presence of $p75^{NTR}$ on follicular dendritic cells was also confirmed. Follicular dendritic cells develop from putative mesenchymal precursors (Van Nierop et al., 2002), they attract and organise lymphoid cells by CXCL13 secretion (Vermi et al., 2008). Recently, $p75^{NTR}$ was established as a marker for human bone marrow mesenchymal stem cells (Álvarez-Viejo et al., 2015). A prominent expression of $p75^{NTR}$ outside of the follicles was also observed along the basal layer of the squamous epithelium. Additionally, in spleen, $p75^{NTR}$ expression was distributed to lymph follicles, along central arterioles, splenic macrophages and endothelial lining at the border of white pulp. The follicular intensity of the immuno-staining shows variations from one follicle to another, probably due to the differences in the germinal centre reaction stage or the three dimensional localization of the section plane.

These findings are in agreement with previous studies (Chesa et al., 1988, Brodie and Gelfand 1992, Pezzati et al., 1992) stating that $p75^{NTR}$ is primarily observed diffusely in the germinal centre cells, i.e. lymphocytes and FDCs (Burton et al., 1993, Hannestad et al., 1995, O. Garcia-Suarez et al., 1997, 1999 and Meada et al., 2002). Dendritic cells have also been reported to express $p75^{NTR}$ (Labouyrie et al., 1997, Bandola et al., 2017).

As $p75^{NTR}$ signaling mainly relies on ligand binding, the next step was to identify a ligand in the tonsil/adenoids. Expression of BDNF but not NGF in lymph follicles has been confirmed on both protein and transcript level. Similar to $p75^{NTR}$, BDNF protein was associated with both B cells and FDCs. Our collaboration partners Berzi et al., reported similar finding in hyperplastic thymus, additionally locating $p75^{NTR}$ on proliferating B cells. BDNF production in lymphoid follicles is of particular importance because Schuhmann et al. reported an impeded Pre-BII stage development in BDNF deficient mice (Schuhmann et al., 2005). In this context BDNF might be an important signaling molecule for B cell or FDC functions in germinal centres. Further analysis is required to determine which form of BDNF is expressed in the follicles, since our protein and transcript detection methods were not able to distinguish between BDNF and proBDNF. Since BDNF is a ligand for both $p75^{NTR}$ and TRKB, expression of TRKB receptor needed to be elucidated.

Immunohistochemical analysis showed that TRKB receptor was mainly expressed in the T cell area, in addition, marginal zone macrophages, FDCs and some lymphocytes, presumably B cells in follicles, were weakly positive as well. To be able to show the localised expression of *p75^{NTR}*, *TRKB* and *BDNF* by quantitative PCR, laser microdissected lymphoid follicles and similar sized T-cell area were analysed. *BDNF* and *p75^{NTR}* transcripts were more abundant in lymphoid follicles whereas TrkB was more prominent in the T cell area. It seems that TrkB is responsible for BDNF signaling outside of the follicles. Coexpression of p75^{NTR} and BDNF in the follicles suggests a ligand dependent function through p75^{NTR}.

Apart from germinal centres in regular lymph follicles, extranodal follicles are found outside of immune organs during various chronic inflammatory conditions including autoimmune diseases, and follicular lymphomas in mucosa associated lymphoid tissue (MALT) or bronchus associated lymphoid tissue (BALT). In this study, extranodal follicles of autoimmune Hashimoto's thyreoiditis and follicular B cell lymphoma were investigated for the expression of p75^{NTR} on protein and transcript levels. Immunohistological analysis showed that p75^{NTR} distribution in extranodal follicles is very similar to regular lymphoid follicles. Quantitative transcript analysis showed that in Hashimoto's thyroiditis, p75^{NTR} expression is significantly higher compared to healthy thyroid controls. We found similar results while assessing the presence of p75^{NTR} in follicular B cell lymphomas. Monoclonal antibody clones C40-1457 and ME 20.4 against human p75^{NTR} provided robust FDC staining comparable with well-established markers CD21/1F8 and CNA.42, thus p75^{NTR} could be used as a marker for healthy lymph follicles as well as autoimmune and malignant follicles. The expression of p75^{NTR} on follicular dendritic cells might have therapeutical implications due to well-known apoptotic function through conserved death domain. Activation of cell-death signal by local delivery of recombinant ligands or mimicking molecules could be a used to eradicate malignant or autoimmune extranodal follicles.

In order to assess *in vivo* functions of p75^{NTR} in the immune system, further experiments were performed using p75^{NTR} knockout mice. This study includes a detailed investigation of p75^{NTR} exon 4 knockout mice for primary and secondary antibody response, memory function, germinal centre reaction and distribution of certain immune cell subsets. The strategy relies on the immunization of mice with three different antigens: Sheep red blood cells (SRBC), ovalbumin (OVA) and virus-like particles (VLP) to observe the changes throughout the humoral immune

response. SRBC and OVA are T cell dependent antigens commonly used for studying antigen specific immune responses in mice. VLPs contain repetitive, high-density displays of viral surface proteins that present conformational viral epitopes that can elicit strong T cell and B cell immune responses (Jegerlehner et al., 2002).

As the main purpose of SRBC immunization was to induce a strong germinal centre response, we focused on histological analysis of the spleen by evaluation of cryosections stained with cardinal markers for relevant cell types. We did not find any remarkable morphological difference in germinal centre formations and distribution of cellular compartments. On the other hand, SRBC immunized $p75^{NTR}$ deficient animals had an increased number of germinal centres and increased total germinal centre area per analysed spleen section, compared to wild type mice. Considering well-established inhibitory role of $p75^{NTR}$ in the nervous system during axonal guidance by growth cone collapse or neuronal cell death, it is possible that lack of $p75^{NTR}$ in follicular dendritic cells or mesenchymal precursor cells might contribute to increased number of germinal centre formation. Following SRBC immunization, a gradual increase was observed in $p75^{NTR}$ mice for B220, IgM and IgD markers by flow cytometric analysis of splenocytes. Although the difference did not reach statistical significance compared to wild type mice, the raise in the number of germinal centres might be reflected to overall B cell counts.

Analysis of serum for antibody production showed that $p75^{NTR}$ deficient mice have similar amount of total IgG and IgM. On the other hand, increased titers of antigen specific IgG and IgM were found compared to wild type mice. Interestingly, antigen specific IgM level were significantly higher for all time points, whereas antigen specific IgG level has reached significance at day 14 only. Immunization with OVA and VLP yielded similar results during primary response. Total IgG, total IgM and antigen specific IgG levels were similar while antigen specific IgM was elevated in $p75^{NTR}$ deficient mice compared to wild type.

Elevated antigen specific IgM levels in $p75^{NTR}$ deficient mice following primary SRBC, OVA and VLP immunizations, suggest a role for $p75^{NTR}$ in B cell compartment. During the primary response, IgM is the first antibody produced by B cells upon exposure to antigen. The cells responsible for the initial production of antigen-specific IgM are splenic plasmablasts that are T-independent, and not found in germinal centres (Papillon et al., 2017). Later come the B cells that have undergone somatic hypermutation and selection but did not switch their isotype from

IgM to IgG or IgA. It is possible that $p75^{NTR}$ plays a role during elimination of less specific Ig-bearing centrocytes or later during class switching, in both case leading to elevated number of mature IgM^+ cells and antigen specific IgM titer.

To test the memory function, mice were challenged with VLP or OVA 60 days after primary immunization. Total and antigen specific serum IgG and IgM levels before and after booster injections were compared. In all cases, antigen specific IgM titer was higher in $p75^{NTR}$ deficient mice both before and after challenge. Existence of significantly elevated antigen specific IgM antibodies after challenge implies:

1- Elevated antigen specific IgM after challenge might be due to the high IgM titer in $p75^{NTR}$ knockout mice before OVA or VLP challenge. It has been reported that administration of antigen specific IgM antibody together with antigen can highly enhance the immune response; this has been shown by immunizing mice with SRBC and anti-SRBC IgM antibodies (Heyman et al., 1982).

2- The possibility of increased number of IgM memory cells or long living IgM plasma cells that have been generated after primary immunization. It is well known that these cells can persist for longer periods maintained in distinct immunological niches, in the absence of immunizing antigen (Maruyama et al., 2000) or supporting cytokines like BAFF (B-cell activating factor) and APRIL (A Proliferation-Inducing Ligand) (Benson et al., 2008). Within these niches, absence of $p75^{NTR}$ may lead to selective activation of Trk receptors on memory B cells, long living plasma cells, supporting epithelial and stromal cells. Concomitant phosphorylation of thyrosine kinases of Trk receptors initiate RAS and MAP kinase pathways, which in turn activate transcription factors promoting maintenance and survival.

3- IgM memory cells reside in the spleen and reinitiate a germinal centre response after challenge (Dogan et al., 2009). IgM memory cells are also present in patients lacking germinal centres, but in reduced numbers, demonstrating partial T cell and germinal centre dependence (Tangye SG et al., 2007, 2009), implicating that more germinal centres yield more IgM memory cells.

Altogether, this is the first study showing that human peripheral blood T cells and NK cells express $p75^{NTR}$, rendering them responsive to NGF family of neurotrophins and proneurotrophins. On the other hand peripheral blood B cells were negative for $p75^{NTR}$. The effects of different neurotrophins on lymphocytes were reported and it is well known that NGF expression is elevated under inflammatory

conditions and lymphocytes are able to secrete NGF and BDNF as well, implicating both paracrine and autocrine signaling in the vicinity.

As for human secondary lymphoid organs, $p75^{NTR}$ was detected in B cells and follicular dendritic cells. Although it was not possible to link $p75^{NTR}$ expression to a single B cell subset with our settings, we detected a gradual expression associated to centrocyte selection and memory B cell maturation, whereas arriving centroblasts and mature plasma cells were negative for $p75^{NTR}$. Strong immunoreactivity of $p75^{NTR}$ and BDNF in germinal centres that is associated to both follicular dendritic cells and B cells and more prominent TrkB expression in T cell area, left $p75^{NTR}$ as the primary receptor for neurotrophins signaling within germinal centres.

In vivo experiments that were performed show that the immune system of the $p75^{NTR}$ deficient mice is fully capable and shows no significant sign of loss of function. Comparable total IgM, total IgG and antigen specific IgG levels upon independent immunizations with three different antigens, indicate that $p75^{NTR}$ signaling has no crucial function in adaptive immune response. Elevated number of germinal centres and antigen specific IgM concentrations indicate for a possible regulation through $p75^{NTR}$.

There is an increasing body of data suggesting that neurotrophins and neurotrophin receptors have key roles in the immune system. They are expressed in the close vicinity of inflammation and immune organs with a potential to trigger molecular mechanisms. It is worthwhile investigating neurotrophin signaling to better understand their role during inflammatory conditions.

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8. Appendices

8.1 Abbreviations

AA	Amino acid
APC	Antigen presenting cell
APC	Allophycocyanin
BALT	Bronchus associated lymphoid tissue
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
BEM	Both ear marking
BMSC	Bone marrow stromal cell
CD	Cluster of differentiation
cDNA	Complimentary DNA
CFA	Complete Freund’s adjuvant
CMV	Cytomegalovirus
CNS	Central nervous system
CRD	Cysteine rich domain
CRR	Cysteine rich region
CT	Cycle treshold
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAPI	4',6-diamidino-2-phenylindole
DD	Death domain
DRG	Dorsal root ganglia
EAE	Experimental autoimmune encephalomyelitis
ECD	Extracellular domain
EGF	Epidermel growth factor
ER	Endoplasmic reticulum
ESC	Embryonic stem cell
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FDC	Follicular dendritic cells
FFPE	Formalin fixed paraffin embedded
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gb	Glycoprotein B
GC	Germinal centre
GM-CSF	Granulocyte macrophage colony stimulating factor
GOI	Gene of interest
HCMV	Human cytomegalovirus
HFF	Human foreskin fibroblasts
HSC	Hemopoietic stem cell
HT	Hashimoto’s Thyroiditis
i.p.	Intraperitoneal
i.v.	Intravenous
ICD	Intracellular domain
IFA	Incomplete Freund's adjuvant
IFNγ	Interferon gamma

Ig	Immunoglobulin
IFNβ	Interferon beta
IP3	Inositol tri-phosphate
IRAK	Interleukin 1 receptor associated kinase
JMD	Juxta-membrane domain
KO	Knock-out
LANGFR	Low affinity nerve growth factor receptor
LANR	Low affinity neurotrophin receptor
LCM	Laser capture microdissection
LEM	Left ear marking
LLPC	Long living plasma cell
LPS	Lipopolysaccharide
LRR	Leucine rich regions
LTP	Long term potentiation
mAbs	Monoclonal antibodies
MAG	Myelin associated glycoprotein
MALT	Mucosa associated lymphoid tissue
MDGI	Myelin derived growth inhibitors
MMP	Matrix metalloprotease
MNCs	Mononuclear cells
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NADE	Neurotrophin associated death executor
NGF	Nerve growth factor
NGFR	Nerve growth factor receptor
NGR	Nogo receptor
NK	Natural Killer
NRAGE	Neurotrophin receptor-interacting MAGE homologue
NRIF	Neurotrophin receptor interacting factor
NT	Neurotrophin
NTBD	Neurotrophin binding domain
NTR	Neurotrophin receptor
NTRK	Neurotropic tropomyosin receptor kinase
OMgp	Oligodendrocyte myelin glycoprotein
OVA	Ovalbumin
PAP	Peroxidase anti-peroxidase
PBMCs	Peripheral blood mononuclear cells
PB	Peripheral blood
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PFA	Paraformaldehyde
PI3-kinase	Phosphodityl-insitol 3-kinase
PKC	Protein kinases C
PLC-γ	Phospholipase C-gamma
PNA	Peanut agglutinin
PNS	Peripheral nervous system
PPIA	Peptidyl-prolyl isomerase A, Cyclophilin A
ProNT	Pro-neurotrophin

PWM	Pokeweed mitogen
qPCR	Quantitative PCR
RA	Rheumatoid arthritis
RBCs	Red blood cells
RG	Reference gene
REM	Right ear marking
RT	Reverse Transcription
s.c.	Sub-cutaneous
sABC	Streptavidin-avidin biocomplex
SAC	Staphylococcus Aurous Cowan 1
SD	Stalk domain
Sd	Standard deviation
SEM	Standard error of the mean
S-p75	p75 short isoform
SRBCs	Sheep red blood cells
TACE	TNFα converting enzyme
TAE	Tris-Acetate-EDTA
TBS	Tris buffered saline
TCR	T cell receptor
TEC	Thymic epithelial cells
TGFβ	Tumor growth factor beta
TLR	Toll-like receptor
TMB	Tetra-methyl-benzidine
TMD	Transmembrane domain
TNFα	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
TNFRSF	Tumor necrosis factor receptor superfamily
TRAF6	TNF recetor assocated factor 6
Trk	The protein tyrosine kinase
VCAM-1	Vascular cell adhesion molecule1
VLP	Virus like particles
WT	Wild type

8.2 Acknowledgements

I am grateful to Prof. Edgar Meinl who supervised my work during all stages of the thesis. He constantly supported and encouraged me with his constructive comments.

I would like to express my gratitude to Prof. Hartmut Wekerle, Prof. Reinhard Hohlfeld and Prof. Edgar Meinl for offering me the opportunity to do my PhD thesis research at the stimulating environment of the MPI of Neurobiology.

I would like to thank Prof. Elisabeth Weiß for her helpful suggestions that added valuable ideas to my study.

My sincere thanks to Dr. Cinthia Farina, for being a congenial colleague and a secondary supervisor who inspired me with her ideas and diligent work. I had numerous motivating discussions with Dr. Markus Krumbholz who has been an outstanding colleague, always being there with the optimal solution.

I want to mention my special thanks to Gurumoorthy Krishnamoorthy for his tremendous help during animal based experiments. I must emphasize indispensable contribution of Prof. Thomas Winkler and Florian Weisel by histological analysis of mice. My thesis would be incomplete without the valuable discussions and samples from Prof. Andreas Rosenwald. And finally many thanks to Dr. Emre Sayan and Dr. Lucia Pinin for kindly providing the p75 knockout mice.

I would like to extend my thanks to Prof. Bettina Kempkes, Prof. Benedict Grothe and Prof. Heinrich Jung for kindly accepting to be in my thesis comity, reviewing and evaluating my thesis.

Many thanks to all members of the MPI Neuroimmunology Department for friendly and relaxing working environment.

I am grateful to my family for their unconditional contribution throughout my studies.

Last, but not least, I would like to express my gratitude to my wife Eser for her support and care.

8.3 Curriculum vitae

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