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Review

Role of neuropilin-2 in the immune system

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ABSTRACT

Neuropilins (NRPs) are single transmembrane receptors with short cytoplasmic tails and are dependent on receptors like VEGF receptors or Plexins for signal transduction. NRPs are known to be important in angiogenesis, lymphangiogenesis, and axon guidance. The Neuropilin-family consists of two members, Neuropilin-1 (NRP1) and Neuropilin-2 (NRP2). They are up to 44 % homologous and conserved in all vertebrates. High levels of NRP2 are found on immune cells. Current research is very limited regarding the functions of NRP2 on these cells. Recent evidence suggests that NRP2 is important for migration, antigen presentation, phagocytosis and cell–cell contact within the immune system. Additionally, posttranslational NRP2 modifications like polysialylation are crucial for the function of some immune cells. This review is an overview about expression and functions of NRP2 in the immune system.

1. Molecular structure of neuropilin-2

Neuropilins (NRPs) were first described in the optic nerve fibers of *Xenopus laevis* and named after the specific area where the antibody A5 binds, called “neuropile” (Takagi et al., 1987).There are two proteins representing the NRP family, NRP1 and NRP2. In humans, they are located on chromosome 10p11.22 for NRP1 and on chromosome 2q33.3 for NRP2. The NRPs are single transmembrane receptors with a predominant extracellular region containing two CUB domains (a1/a2), two Factor V/VIII homology domains (b1/b2), and a MAM domain (c) (Fig. 1). NRP1 and NRP2 can form homodimers as well as heterodimers. Both NRPs are highly conserved in vertebrates and the sequence homology of human and murine NRP2 is 94%. NRP1 and NRP2 are up to 44% homologous (Rossignol et al., 2000). Both molecules are heavily glycosylated. Additionally, NRP2 is one of the few proteins which can be sialylated (Curreli et al., 2007). The NRP1 and NRP2 have different splice variants which are between 551 and 926 amino acids in length. Two major variants exist in the case of NRP2, and are categorized as NRP2a and NRP2b. These differ in their intracellular C-terminal part (Fig. 1). In detail, the short C-terminally located cytoplasmic tail of NRP2a consists of 42 amino acids and has a PDZ-binding-domain with the C-terminal SEA amino acid sequence. The C-terminus of the other isoform, NRP2b, consists of 46 amino acids and shares just about 11% of the intracellular and transmembrane sequence of NRP2a (fig. 1).

In mice, NRP2a can have insertions after amino acid 809 and the resulting variants are named according to the number of added amino acids, NRP2a (0), (5), (17) and (22). NRP2a (17) is generated by alternative splicing between exon 15 and 16. For NRP2a (22) a different splice acceptor is active at intron 15 and 5 amino acids (GENFK) are added without shifting the reading frame (Fig. 1). Four splice variants for NRP2a were described but Rossignol et al. found only two isoform bands in lung, brain, heart, kidney, liver, placenta, and trachea in their PCR for NRP2a corresponding to NRP2a (17) and NRP2a (22). They wereńt able to identify NRP2a (0) and NRP2a (5) in humans, which would be generated by splicing at exon 16, but the theoretical sequences can be found on PubMed. The theoretical human sequence for NRP2a (0) was used in a study from Rollenhagen et al., although it has never been identified in human tissue and cells at this point (Rollenhagen et al., 2013; Rossignol et al., 2000). It is possible that NRP2a (0) and NRP2a (5) are expressed in certain tissue or cells, but a more detailed analysis needs to be performed to shed light on the expression pattern of the splice variants of NRP2.

Rossignol et al. identified an exon sequence in intron 15 named exon 16b. It consists of 912 bp in total with a coding sequence of 283 bp, a stop codon and a 3′UTR including a cleavage and polyadenylation signal. Therefore, the alternative splicing for NRP2b results most likely between exon 15 and exon 16b. Two splice variants can occur, NRP2b (0) and NRP2b (5) depending on the insertion of 0 or 5 amino acids (GENFK) after amino acid 808 as described for NRP2a (Fig. 1) (Rossignol et al., 2000).

The difference in the C-terminal sequence, more specifically the PDZ domain, between the splice variants might result in different binding capabilities to other proteins. One protein with repetitive PDZ domains is GAIP interacting protein 1 (GIPC1)/Synectin. In fact, GIPC1/Synectin

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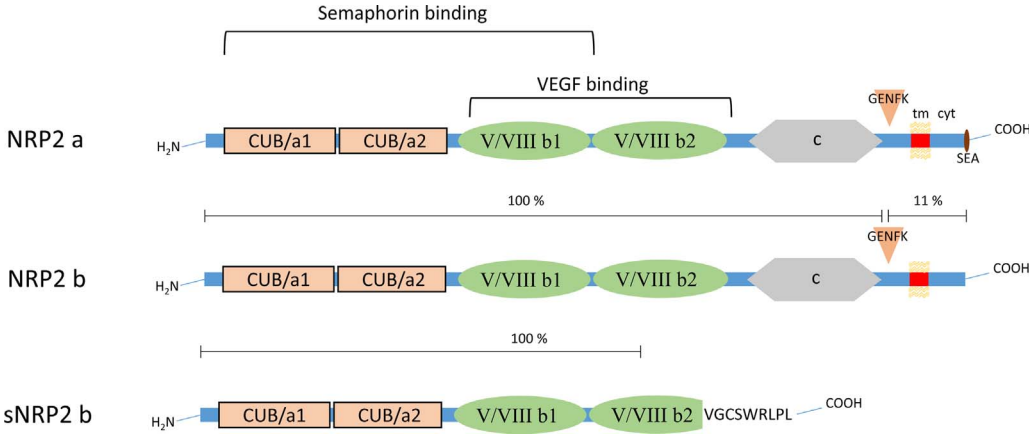

Fig. 1. Splice variants of Neuropilin 2. Neuropilin 2 consists of two CUB domains followed by two Factor V/VIII domains followed by a MAM (c) domain for both NRP2a and NRP2b. These two are the main isoforms in human and mice. Between the MAM domain and the membrane (tm) additional splicing can occur and 5 additional amino acids (GENFK) can be added to NRP2a and NRP2b resulting in two different variants for NRP2a (17), (22) and two different transmembrane variants for NRP2b (0), (5) as well as one soluble form called sNRP2b. For mice, the following variants have been identified: NRP2a (0), (5), (17), (22) and NRP2b (0), (5).

binds to the C-terminal SEA domain and could be an initial trigger for a direct signal (Muders et al., 2006, 2009; Prahst et al., 2008). Furthermore, GIPC1/Synectin might have an important function as a scaffold for multiprotein complexes. Others and we have already shown that GIPC1/Synectin has important biological functions (Gao et al., 2000; Muders et al., 2009; Naccache et al., 2006). NRP2a is preferentially expressed in the brain, liver, lung, small intestine, kidney and heart while NRP2b is mainly expressed in the heart and muscles (Rossignol et al., 2000).

2. Binding and interaction partners

The group of soluble semaphorin 3 (SEMA 3) proteins and vascular endothelial growth factors (VEGFs) are known binding partners for the NRP family. NRP1 and NRP2 have different affinities to bind various semaphorins or VEGFs. Especially, SEMA3A binds to NRP1 whereas SEMA3F and SEMA3C bind to NRP2. VEGF-A binds to NRP1 and NRP2 but the affinity depends on the splice variants of VEGF-A. VEGF-C is an important binding partner for NRP2 (reviewed in (Prud'homme and Glinka, 2012)).

Semaphorins and VEGF have different binding domains and therefore are no competitors for binding to NRPs (Appleton et al., 2007). Also, it is well established that SEMA3 and VEGF binding to NRPs leads to dissimilar effects in the cell suggesting that different signal cascades become activated through binding of each ligand. SEMA3 are involved in vascular permeability, inhibiting of endothelial cell proliferation, and can induce apoptosis, whereas VEGFRs are involved in vasculogenesis and angiogenesis.

Another interaction partner for NRP2 is PTEN. PTEN is important for the inhibiting effect of the Semaphorin3F/NRP2 axis in some cells like T lymphocytes. In these cells, PTEN knock-down eliminates the inhibiting effect of Semaphorin3F (Nakayama et al., 2015).

A more detailed overview about the binding partners of NRP1 and NRP2 were reviewed 2012 from Prud́homme and Glinka (Prud'homme and Glinka, 2012).

NRPs are known for their crucial function in neurogenesis and cardiovascular development as it was shown in different animal models. For example, a full knock-out of NRP1 leads to death at embryonic day E10–E13.5 with a spectrum of cardiovascular defects (Takashima et al., 2002b). A knock-out of NRP2 is not lethal but displays abnormal guidance of nerves and fewer small vessels and capillaries. Double knock-out of NRP1 and NRP2 leads to an even earlier death at E8 and has a similar phenotype like a VEGF-A and VEGFR2 knock-out and therefore underlines the importance of the interactions between VEGFs and NRPs (Takashima et al., 2002a).

3. Posttranscriptional modification of NRP2 by ST8Sia IV

NRP2 and to a much lesser extent NRP1 are two of the few proteins which can be posttranslationally modified by polysialylation (Bhide et al., 2016; Mühlenhoff et al., 1998). To our knowledge only six other proteins in mammals can carry polysialylation. The neuronal adhesion molecule (NCAM/CD56) was first described in 1982 to carry polysialylations. Many researchers studied it during the years and were able to point out the importance of polysialic acid (polySia) (Finne, 1982) (reviewed in (C. E. Galuska et al., 2017)). Other polysialylated proteins are: the cluster of differentiation (CD) 36 (mouse/human) (Yabe et al., 2003), C-C chemokine receptor type 7 (CCR7) (mouse/human) (Kiermaier et al., 2016), E-selectin ligand-1 (ESL-1) (mouse/human) (Werneburg et al., 2016), Synaptic cell adhesion molecule SynCam-1 (mouse) (S. P. Galuska et al., 2010) and the sodium channel α subunit (rat) (Zuber et al., 1992).

The CMP-N-acetylneuraminate-poly-alpha-2,8-sialyltransferase (ST8Sia IV) is one of two enzymes facilitating sialylation and is uniquely responsible for the addition of the sialic acid to NRP2. The other enzyme is called alpha-2,8-sialyltransferase 8B (ST8Sia II) and is not involved in the modification of NRP2 (Curreli et al., 2007). Addition of sialic acids can occur in a *N*-linked or *O*-linked manner. In case of NRP2, the sialylation is restricted to the linker region and can contain up to 100 α2,8-linked sialic acid residues but has at least 8 residues (Rollenhagen et al., 2013; Sato and Kitajima, 2013). Despite 3 possible *N*-glycosylation sites and 2 *N*-glycans at Asn-152 and Asn-157, NRP2 carries only *O*-linked polysialylation (Rollenhagen et al., 2013). In the linker region, 6 possible sites for *O*-glycosylation and sialylation exist but the major, if not exclusive, sites are Thr 613, Thr 614, Thr 615 and Thr 619 (Rollenhagen et al., 2013) (Fig. 2). The sialylation varies between the different splice forms of NRP2. NRP2b and especially NRP2b (5) is more sialylated than NRP2a (Rollenhagen et al., 2013). Therefore, Bhide et al. elucidated the responsible domain for sialylation. They found that a replacement of the MAM domain of NRP2 by the MAM domain of NRP1 reduced the polysialylation of NRP2 significantly. Furthermore, they identified the Glu^{652} and Glu^{653} in the MAM domain as key elements for sialylation by ST8SiaIV (Bhide et al., 2016).

The polySia can bind to chemokines like CCL21 and the receptor CCR7 and is involved in migration. Other functions of the polySia are anti-adhesion, development of the nervous system, cell–cell and cell-matrix interaction, phagocytosis, and T-cell activation (Bhide et al., 2016; Drake et al., 2009; Rutishauser, 2008; Stamatos et al., 2014).

In this review, we focus on the expression and known function of NRP2 in immune cells.

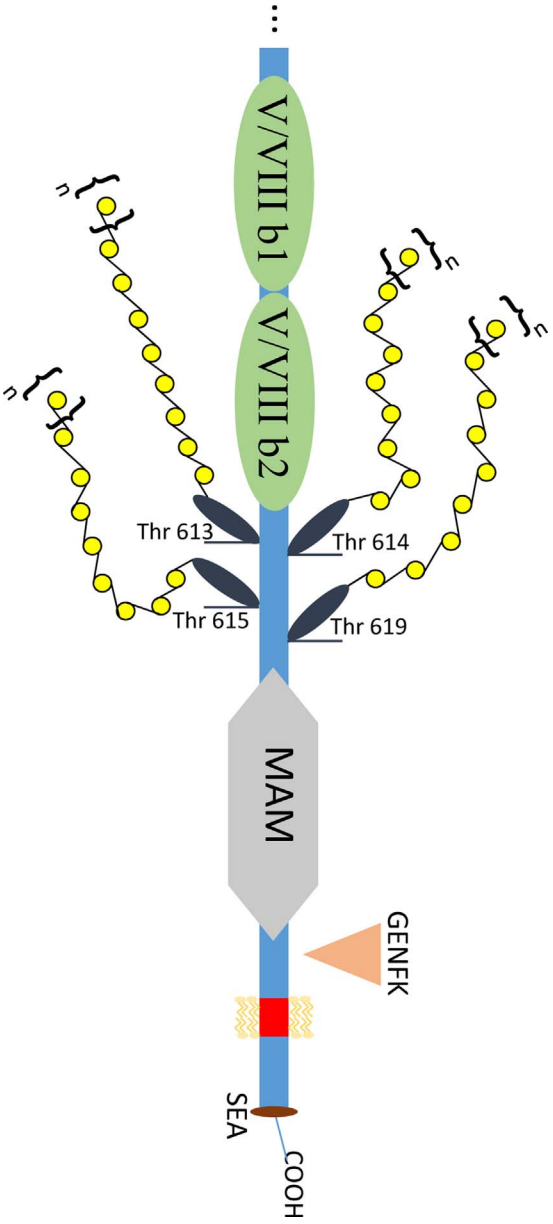

Fig. 2. NRP2 with 4 sialic acids at Thr 613, Thr 614, Thr 615 and Thr 619.

4. Neuropilin-2 expression in the immune system

4.1. Myeloid lineage

NRP2 is expressed in many cells of the immune system but not in every differentiation state (Fig. 3). NRP2 is not detectable in the bone marrow or monocytes of mice or humans (Ji et al., 2009; Stamatos et al., 2014) and (Stepanova et al., 2007a; Verlinden et al., 2013). During the differentiation from human and murine monocytes to macrophages the cells start to express NRP2. Stamatos et al. isolated and cultured peritoneal exudate cells (PECs). These cells differentiated towards macrophages and upregulate NRP2 (Stamatos et al., 2014).

Macrophages polarize further and are classically divided into two major populations: M1 and M2 macrophages. M1 macrophages are known for their pro-inflammatory character whereas M2 macrophages are characterized by a more anti-inflammatory profile (Martinez and Gordon, 2014).

So far, Ji et al. published a reduced expression of NRP2 after LPS stimulation of macrophages that triggers M1 polarization (Ji et al., 2009). In experiments of this group only a very short culture time is used. Accordingly, the validity of these statements is questionable. In contrast, Werneburg et al. showed NRP2 expression in M1 macrophages (Werneburg et al., 2016). Concerning NRP2, the M2 macrophage is under investigated. In cell culture, M-CSF treated monocytes showed an upregulation of NRP2 (Ji et al., 2009).

In tissue specific macrophages (microglia in the brain, Kupffer cells in the liver, red pulp macrophages in the spleen and sinus histiocytes in the lymph node) of human patients, Aung et al. were able to show expression of NRP2 (Aung et al., 2016). Such a broad screening does not exist for tissue specific macrophages in the mice, but RNA and protein data is available for microglia (population of macrophages in the brain) (Werneburg et al., 2015). Furthermore, Aung et al. analyzed the frequency of NRP2 positive alveolar macrophages (AM) regarding their position towards an inflammation. The highest frequency of NRP2 expression was in AMs adjacent to the cancer margin, followed by macrophages in inflamed lungs, and at least frequency in tissue furthest away from the cancer. (Aung et al., 2016). These studies elicited the question if the expression of NRP2 is affected by the origin of the macrophage. Also, this data indicates a correlation of NRP2 expression and function in macrophages. Accordingly, macrophages in close proximity of an inflammatory response demonstrate a higher frequency of NRP2 expression.

Due to the broad and strong expression of NRP2 in many macrophages in humans and mice, it is likely that NRP2 has an important function in these cells. Interestingly, posttranslational modifications are an important prerequisite for these immune specific functions. One possible modification is the polysialylation of NRP2 in macrophages and microglia. Among other functions polySia is known to be involved in certain cell types in migration, phagocytosis, antigen-presentation, cell–cell and cell-matrix contact (Bhide et al., 2016; Drake et al., 2009; Rutishauser, 2008; Stamatos et al., 2014; Werneburg et al., 2015). Phagocytosis especially is a very important mechanism in macrophages for clearance of pathogens and cells. Interestingly, removal of polySia leads to an increase of phagocytosis in macrophages (Stamatos et al., 2014). Matured macrophages from inflamed sites lose the polysialylation but can re-express polySia on NRP2 after 24 h of culture and therefore could be important for the migration towards inflammation (Stamatos et al., 2014). One hypothesis is that polySia might inhibit the function of NRP2 in phagocytosis. Recently, we were able to show that NRP2 is needed for endocytosis in cancer cells. This further supports the hypothesis that NRP2 is important for the phagocytosis in macrophages and might be inhibited when polysialylated (Dutta et al., 2016).

Macrophages are not the only cells of the myeloid lineage that express NRP2, but not all the different cells have been analyzed for their expression of NRP2 yet. DePaulis et al. were able to show that human basophiles express NRP2 and Vales et al. found NRP2 in mast cells of leukemia patients (de Paulis et al., 2006; Vales et al., 2009).

Dendritic cells (DCs) can originate either from myeloid lineage or from lymphoid lineage and are the main antigen presenting cells. Therefore, they are especially known for phagocytosis of pathogens, migration toward the lymph node, and presentation and activation of T cells (Alvarez et al., 2008). During the differentiation from monocytes towards DCs the cells start to express NRP2. It highly increases during the maturation process. In contrast, NRP1 decreases during the maturation which indicates that NRP1 and NRP2 most likely have different roles in DCs.

Posttranslational sialylation is essential for the function of NRP2 on DCs. Curreli et al. postulated that polysialylation of NRP2 acts as a protective shield around the activated DCs during their migration to lymph nodes, thereby preventing interaction with other cells on their way. But upon reaching their destination in the lymphatic tissue the polysialic acid is removed and NRP2 can modulate the activation of T cells (Fig. 4). Additionally, it is already known that the polysialic acid is also involved in migration of endothelia and tumor cells (Geretti et al., 2008; Pellet-Many et al., 2008). Therefore, polySia NRP2 might not

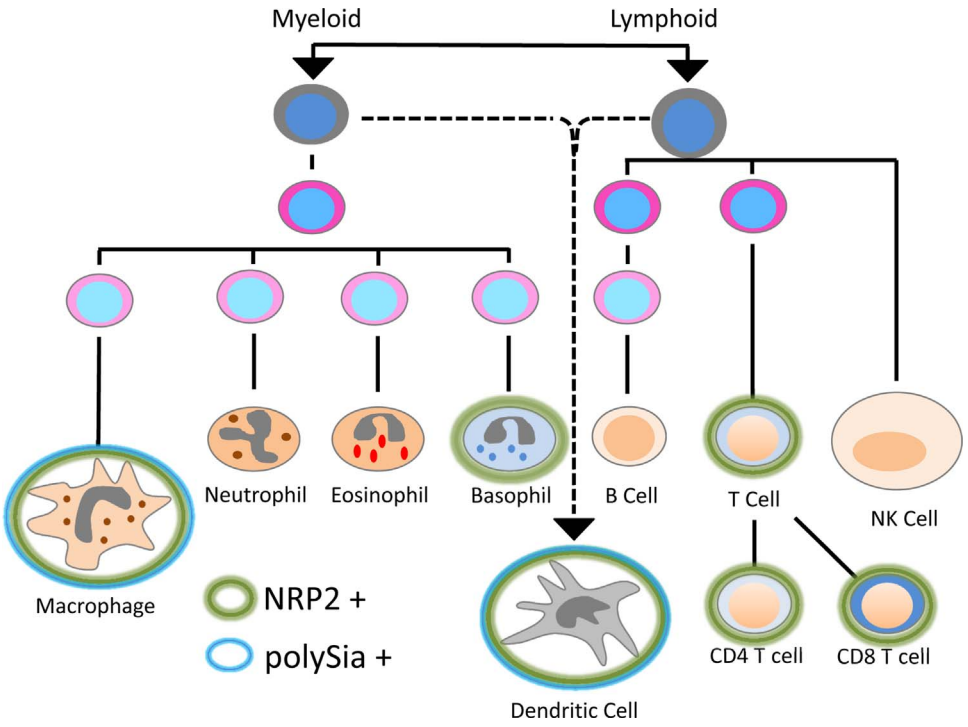

Fig. 3. Cells of the immune system. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
Macrophages, dendritic cells and T cells, CD4 and CD8T cells have high levels of NRP2 as indicated by a green circle. Another known NRP2 positive cell type are basophils. The blue circles mark cells which can carry polysialic acid.

only work as an inhibitor of immune system activation on DCs but could also be involved in migration. Indeed, chemotactic migration towards the CCL21/CCR7 axis in DCs is dependent on polysialylated NRP2. Without polysialylation of NRP2, the binding of CCL21 and the activation of JNK and Akt through CCL21/CCR7 pathway is impaired. This observation suggests that the polySia on NRP2 can capture and trap CCL21. That leads to an increased concentration of CCL21 on specific regions of the cell membrane and optimally aids in directed migration of DCs. (Rey-Gallardo et al., 2011, 2010).

4.2. T cells – lymphoid lineage

No data is currently available for the expression pattern of NRP2 in B-cells, neutrophils, eosinophils or NK cells, yet (Fig. 3). Many papers addressed the role of NRP1 in T cells, especially in Treg cells, but only very few authors focused on NRP2 in T lymphocytes. In 2007, *Stepanova* et al. first published the expression of NRP2 in mice thymocytes and lymph nodes (Stepanova et al., 2007b). A recent paper showed a more detailed expression pattern for NRP2 in human T cells. They divided thymocytes according to their expression into CD8 (cytotoxic T) and CD4 (T helper) cells. Because the expression of CD4 and CD8 decreases during maturation of T cells, Mendes-da-Cruz et al. distinguished between high, low and no expression of these receptors (Mendes-da-Cruz et al., 2014).

Mendes-da-Cruz et al. used T cells from young humans and studied the cells by flow cytometry analysis. Only a very small percentage of CD4 and CD8 negative T cells express NRP2, whereas nearly all double positive T cells do. The population of T cells with a low expression of CD4 display less NRP2 on their surface than the CD4 high population. The same applies for the CD8 cells. It is important to note, that nearly all cells of the CD4 high and CD8 high population are NRP2 positive (Mendes-da-Cruz et al., 2014).

Analysis of the subpopulation of T cells indicates that NRP2 might be important for some T cells during their maturation process in a very stage-dependent manner. A more detailed investigation of the CD8 positive and CD4 positive populations would be beneficial to explore

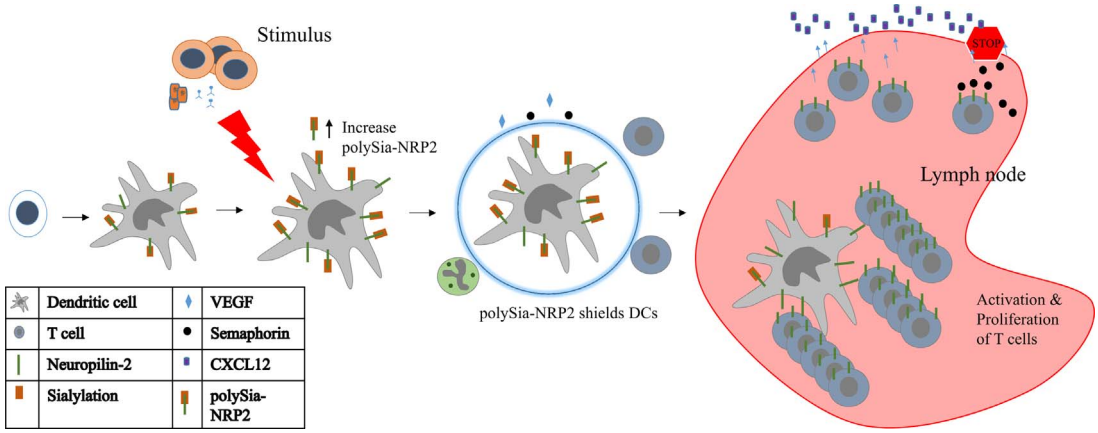

Fig. 4. Function of NRP2 in the migration and activation of T cells via DCs.
During the differentiation of monocytes to DCs NRP2 is upregulated. After an activation of the DCs NRP2 is further upregulated and sialylated. The sialylation might shield the DCs from other stimuli on the way towards the lymph node. In the lymph node, sialylation decreases and the DCs activate T cells. The migration of T cells out of the lymph node can be inhibited through the binding of semaphorins on NRP2.

the role of NRP2 in T cells.

One of the best described functions of NRP2 is migration. Mendes-da-Cruz et al. studied the function of Semaphorin3F binding to NRP2 on migration in thymocytes. They were able to show that Semaphorin3F has a repulsive effect on the migration of these cells and inhibits the migration towards CXCL12 and sphingosine-1-phosphate (S1P) by inhibition of the cytoskeleton remodeling prior to the stimuli (Mendes-da-Cruz et al., 2014).

In this review, we summarized the differential expression and functions of NRP2 in the immune system. There are many open questions about the function of NRP2 in immune cells: Why and how NRP2 is regulated during the differentiation and maturation of macrophages and DCs remains unclear. Why is NRP2 high expressed in some T cells while others seem to downregulate NRP2? Do other cells in the immune system express NRP2 and during what stages of differentiation? What is the function of NRP2 in basophils?

We believe that during the next couple of years new exciting functions of NRP2 in different cells throughout the immune system will be published and new fields to study NRP2 will emerge. NRP2 holds a high potential to be an important player in some T cells, macrophages and DCs with novel functions awaiting to be revealed.

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