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Dectin-1 and Langerin: Dissecting C-Type Lectin Receptor Signaling in Glycan-Mediated Immune Responses

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ABSTRACT

Pilz-Beta-Glukane aktivieren die Immunantwort über verschiedene Rezeptoren, darunter C-Typ-Lektin-Rezeptoren (CLRs). Eine besondere Rolle spielt dabei Dectin-1. Frühere Studien deuteten darauf hin, dass der Dectin-1-Signalweg, der zur Interleukinproduktion führt, nur durch Hefepartikel aktiviert werden konnte, indem eine Immunsynapse gebildet wird. Die Arbeit zeigt jedoch, dass kurze lösliche Fragmente wie tetrametere Beta-Glucane auch den NF-kB- und MAPK-Signalweg aktivieren können. Mit Nanopartikeln, die Hefepartikel nachahmen sollen, konnte ich die Dichte definierter Glukane manipulieren und den Schwellenwert für die Aktivierung der Dectin-1-Signale bestimmen.

Der Schwerpunkt liegt auch auf der Erkennung bakterieller Glykane, insbesondere auf der Interaktion von Dectin-1 und Langerin mit Wandteichonsäuren (WTAs) von Staphylococcus aureus und Streptococcus pyogenes. Durch die Erzeugung von Bakterienstämmen mit spezifischen Modifikationen der WTA-Glykosylierung (z.B. ΔtarM, ΔtarS, ΔtarP) konnte ich wichtige Glykosyltransferasen identifizieren, die für die Veränderung der Immunerkennung verantwortlich sind. Dies gibt Aufschluss darüber, wie bakterielle Zellwandänderungen die Interaktion zwischen Wirt und Erreger beeinflussen, was wiederum Auswirkungen auf das Verständnis der bakteriellen Immunantwort hat. Durch den Einsatz von zellbasierten Assays und hochauflösenden Bildgebungsverfahren (TIRF) stellt diese Arbeit einen guten Ansatz dar, um der Erforschung der intrazellulären Kommunikation mit Glykanen eine neue Dimension zu eröffnen.

ABSTRACT

Fungal beta glucans are known to activate the immune response through various receptors, including C-type lectin receptors (CLRs). Notable, among these receptors, Dectin-1 stands out for its crucial role in fighting fungal infections. Despite efforts to fully understand Dectin-1 signaling, the precise molecular mechanisms remain unknown. Previous research suggested that Dectin-1 signaling leading to interleukin production could only be activated by yeast particles through the formation of an immune synapse that requires phase-segregation of CD45 phosphatase and clustering of Dectin-1 receptor. In this work, however, I demonstrate that short soluble fragments like tetrameric beta-glucans can also activate the NF-kB and MAPK pathway. Using silica particles to mimic yeast particles, I was able to manipulate the density of defined glucans and determine the threshold for activating Dectin-1 signaling.

This work also focuses on the recognition of bacterial glycans, specifically on the interaction of Dectin-1 and Langerin with wall teichoic acids (WTAs) of Staphylococcus aureus and Streptococcus pyogenes. By generating bacterial strains with specific deletions and modifications in WTA glycosylation (e.g., ΔtarM, ΔtarS, ΔtarP), we identified key glycosyltransferases responsible for altering immune recognition. We show that modified bacterial glycans activate CLR signaling, with the potential to modulate the immune response. This sheds light on how bacterial cell wall modifications affect host-pathogen interactions, with implications for understanding bacterial immune evasion. By employing both cell-based assays and high-resolution imaging techniques (TIRF), this work represents a unique approach to provide a new dimension to explore intracellular communication through glycans.

ZUSAMMENFASSUNG

Die Arbeit untersucht die Rolle von C-Typ-Lectin Rezeptoren (CLRs) bei Immunreaktionen, insbesondere deren Wechselwirkung mit Beta-Glukanen. Die Studie konzentriert sich auf Dectin-1, ein CLR, die Beta-Glukane erkennt und Immunwege, wie NF-kB und MAPK/ERK aktivieren kann. Die Studie zeigt, dass lösliche Beta-Glukane diese Wege aktivieren können. Die Arbeit untersucht auch die Verwendung von humanem Langerin, einem weiteren C-Typ Lectin Rezeptor, für mögliche Vakzinanwendungen.

Unter Verwendung hochauflösender Bildgebung sowie mehrerer zellbasierter Assays mit Reporterzelllinien deuten die Ergebnisse auf therapeutisches Potenzial hin, wenn es darum geht, Dectin-1-Wegwege zur Behandlung von Pilzinfektionen anzuvisieren und Langerin für innovative Arzneimittelabgabesysteme. Die Masterarbeit trägt dazu bei, zu verstehen, wie C-Typ Lectin Rezeptoren Immunreaktionen vermitteln, und öffnet neue Wege für medizinische Anwendungen.

ACKNOWLEDGEMENT

First of all, I want to thank Prof. Rademacher for this opportunity to do my master thesis in his lab. It gave me the opportunity to strengthen my skills in cell culture, but also to learn new techniques and dive into the world of cell imaging. The atmosphere in the team was always amazing and inspiring, and even with the occasional banter everyone focused on their work.

A special thank you goes out to Anna and Giada, who made the late nights in the lab way more enjoyable. Not only did I find good labmates, but also good friends along the way. Last but not least, a little special thank you to Gabriele, who never failed to make me laugh, even when my experiments did not go as planned – I definitely miss the Italian spirit.

I had an amazing year in this lab and I miss all of them!

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1. Introduction

1.1 Innate Immune system

The immune system is divided in two parts: innate and adaptive immune system. The innate immune system is also often referred to as the “first line of defense against pathogens, such as viruses, bacteria, and fungi”. It is called "innate" because it is present from birth and does not change throughout a person's life, in contrast to the adaptive immune system, which develops over time and can adapt to specific pathogens. (Alberts et al., 2002). The innate immune system includes several different components, such as physical and chemical barriers, as well as cells that can recognize and respond to pathogens. Some examples of physical barriers include the skin and mucous membranes, which can prevent pathogens from entering the body (Alberts et al., 2002). One of the main functions of the innate immune system is to present antigens via antigen presenting cells (APC) and thus activate the adaptive immune system. Cells of the innate immune system include macrophages, neutrophils, and dendritic cells. Macrophages and neutrophils can ingest and kill pathogens, while dendritic cells act as messengers, alerting the adaptive immune system to the presence of a pathogen (Peter J. Delves, 2022)

Moreover, those cells mentioned above express pattern recognition receptors (PRR), which are able to detect pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pattern (DAMPSs) (Tang et al., 2012). PRRs can be further divided in groups.

There are several different classes of PRRs, each of which can recognize different types of PAMPs. Some of the main classes of PRRs include:

- Toll-like receptors (TLRs): TLRs are found on the plasma membrane of many different types of cells, including macrophages, dendritic cells, and B cells. There are

  currently 13 known human TLRs, each of which can recognize different PAMPs (Takeda & Akira, 2005). For example, TLR4 can recognize lipopolysaccharide

(LPS), a component of the outer membrane of gram-negative bacteria, while TLR3 can recognize double-stranded RNA, a characteristic of some viruses (Chen et al., 2021; Molteni et al., 2016).

- Nucleotide-binding oligomerization domain-like receptors (NLRs): NLRs are similar to TLRs in that they can recognize PAMPs and trigger an immune response. Unlike TLRs, however, NLRs are found inside the cell, and can recognize PAMPs that have entered the cell. NLRs can also respond to "danger signals" produced by the host cells, such as damage-associated molecular patterns which is an indication of infection, injury or stress (Zhong et al., 2013).
- RIG-I-like receptors (RLRs): RLRs are receptors that specifically recognize viral RNA. RIG-I and MDA5 are two examples of RLRs, they both have a similar function of binding to the viral RNA present in the cytoplasm and triggers the immune response (Rehwinkel & Gack, 2020).
- C-type lectin receptors (CLRs): CLRs are a diverse family of receptors that can mainly bind to the glycans found on the surface of microorganisms. CLRs include Dectin-1, which can bind to beta-glucans, a component of the cell walls of many types of fungi, as well as Dectin-2. (Mayer et al., 2017).
- Other PRRs: There are other PRRs as well, such as retinoic acid-inducible gene-I (RIG-I), absent in melanoma 2 (AIM2) and stimulator of interferon genes (STING).

This work will focus on C-type lectin receptors, more specifically on Dectin-1, thus in the following chapters, this lectin family will be discussed in detail.

1.2 C-type lectins

To distinguish between carbohydrate-binding lectins that are Ca2+ dependent and those that are Ca2+ independent, the name C-type lectin was developed (G. D. Brown et al., 2018; Geijtenbeek & Gringhuis, 2009). C-type lectin receptors (CLRs) are a

large superfamily of over 87 proteins in humans and represent the largest group of glycan-binding proteins. Their defining feature is the carbohydrate recognition domain (CRD), a structural module with conserved residue patterns that determine the receptor's carbohydrate-binding specificity. Since not all proteins containing this domain bind glycans or Ca2+, the CRD of CTLs is more widely referred to as the CTL domain (CTLD) (Cummings & McEver, 2009; Weis et al., 1998). In total, they are divided into 16 subgroups ((Drickamer & Fadden, 2002), including proteoglycans, tetranectins and selectins. Various C-type lectins are expressed by cells of both the innate and adaptive immune systems, including all myeloid cells and lymphocytes. Among these, some are commonly expressed by leukocytes, such as L-selectin (Ivetic, 2013).

Myeloid cells, including dendritic cells and macrophages, express a large number of C-type lectins that are involved in endocytosis of ligands. Endocytosis of ligands in those cells can result in receptor accumulation and degradation in phagolysosomes, or recycling of the receptor back to the cell surface. The specific pathway taken is determined by the type of ligand bound by the receptor (McGreal et al., 2004).

Several receptors within this family bind to different ligands, displaying different parts in the innate immune system. One such receptor that belongs to CTL is Dectin-1, which is recognized for its involvement in recognizing β-glucans found in fungal cell walls. Another important player is the mannose receptor, which, as its name suggests, binds to mannose-containing ligands commonly found on pathogens.

The classification of CTL depends on their domain architecture (Fig. 1). However, they all share common structural features. All of them have at least one carbohydrate recognition domain (CRD, also known as CTLD) which has conserved motifs. The CTLD structure is defined by a distinctive double-loop formation, often referred to as a 'loop-in-a-loop, anchored by two highly conserved disulfide bridges at the bases of the loops. It also features hydrophobic and polar contacts (Zelensky & Gready, 2005).

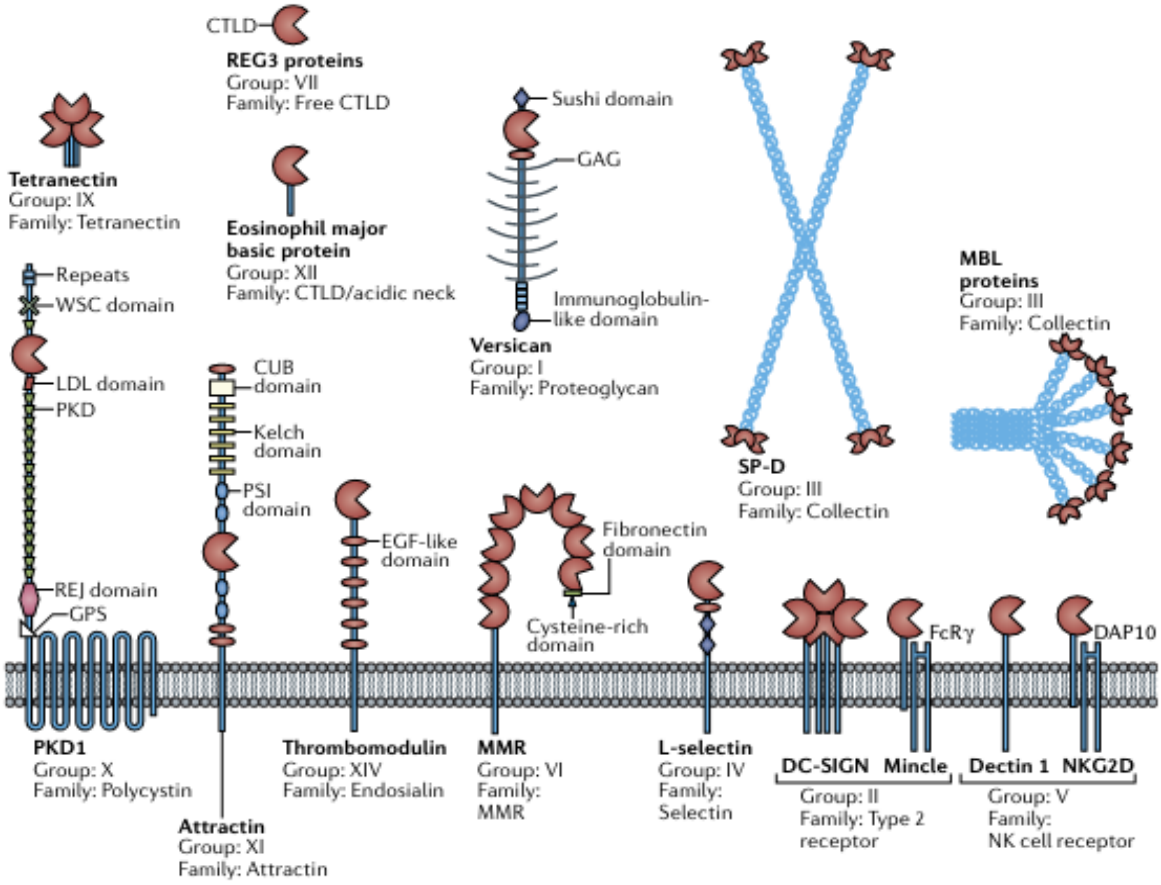

Figure 1: Classification of different C-type lectins. The major soluble and membrane-bound C-type lectins are depicted in this figure. They can be divided into 17 subgroups depending on their domain organization, but all of them have at least one CTLD. (Figure adapted from: Brown et al. 2017)

C-type lectins can form homodimers, homotrimers, and higher-ordered oligomers, that increases their affinity for multivalent ligands. Despite sharing structural homology, these proteins typically differ significantly in their ability to recognize specific types of glycans with high affinity (Cummings & McEver, 2009). They can be also classified them into two groups: soluble CLRs and transmembrane CLRs (Fig. 1). The transmembrane group can be further divided into type 1 CLRs, also known as the mannose receptor family, and type 2 CLRs, known as Asialoglycoprotein receptors (this depends on the orientation of their N-terminus) (Mayer et al., 2017). Type I C-type lectin-like receptors (CTLs) typically possess multiple carbohydrate recognition domains (CRDs) that also contain a CTLD domain, whereas type II CTLs are characterized by a receptor structure consisting of a single CRD (Figdor et al., 2002).

The transmembrane CTL group Asialoglycoprotein contains Selectins, DC-SIGN, Mincle, Dectin 1 and more. Both groups of transmembrane receptors are receptors for pathogen recognition and act as a pathogen recognition receptor (PRR), similar to Toll-Like receptors (TLR). The pathogen is internalized after being recognized by CLRs, whereupon it is degraded and then presents its antigens (van Kooyk & Rabinovich, 2008).

This work will focus mainly on two C type lectins, namely Dectin-1 and Langerin.

1.3 Dectin-1

Dendritic cell associated C-type lectin 1 (Dectin-1, also known as CLEC7A) belongs to the class of myeloid-cell- expressed natural killer (NK)-cell-receptor-like C-type lectin family. Dectin-1 shares structural similarities with the other members of the NK-like C-type lectin family. It contains a single extracellular C type lectin like domain (carbohydrate-recognition domain, CRD), which can recognize beta glucans. The stalk region separates the CRD from the membrane (Figure 2). In the cytoplasmatic region it contains an ITAM motif as well (Brown et al., 2002). Dectin-1 does not have the Ca^{2+} binding site in its CRD, therefore, ligand recognition is Ca^{2+} independent. Since Dectin-1 lacks the cysteine residues necessary for dimerization in the stalk region, the receptor seems to express and work as a monomer (Mata-Martínez et al., 2022; Tsoni & Brown, 2008). Dectin-1 receptor is predominantly expressed by myeloid cells such as macrophages, dendritic cells, and neutrophils in mice, although it has also been detected on a subset of B and T lymphocytes through flow cytometry. However, the function of Dectin-1 on these lymphocytes is still unknown (Noorbakhsh Varnosfaderani et al., 2024; Willment et al., 2005).

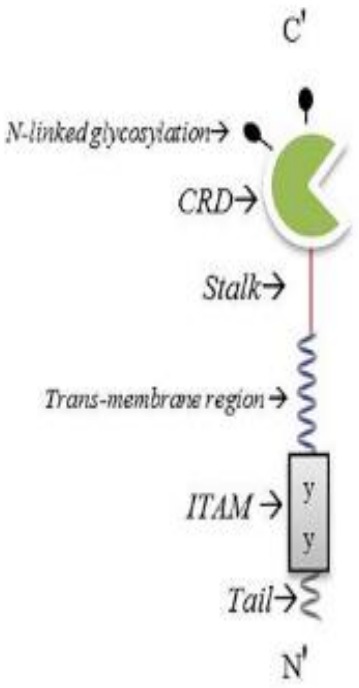

Figure 2: Full length Dectin-1. Dectin-1 possesses a CRD (carbohydrate recognition domain), a stalk (which separates the CRD from the membrane, and a trans-membrane region. After that, a hemi-ITAM motif in the cytoplasmatic region can be found at the N-terminus. (Figure adapted from: Kalia et al., 2021)

1.3.1 Structure

Unlike murine Dectin-1, human monocyte populations express two major isoforms of Dectin-1, A and B, which differ in the presence of a stalk region and an N-linked glycosylation site. Moreover, due to alternative splicing, several minor isoforms exist (Fig. 3) (Fischer et al., 2017).

Isoform A has the full-length structure, including an extra-cellular carbohydrate recognition domain, a stalk, a trans-membrane region, and a tail with an immune receptor tyrosine-based activation motif (Fig. 3). Isoform B is similar to isoform A but lacks a stalk. Only those two can bind zymosan particles containing beta-glucans, mannan, and proteins. Isoforms C and D have deletions in the carbohydrate recognition domain. Isoforms E and F lack the stalk and trans-membrane region. Isoforms G and H have insertions in the carbohydrate recognition domain and trans-membrane region. All of the minor isoforms, except E, express truncated proteins due to the presence of premature stop codons. Isoform E is expressed in the cytoplasm and interacts with other cytosolic proteins, and is thought to have regulatory roles for

the major isoforms. The functions of the other minor isoforms are currently unknown.(Heinsbroek et al., 2006; Willment et al., 2001).

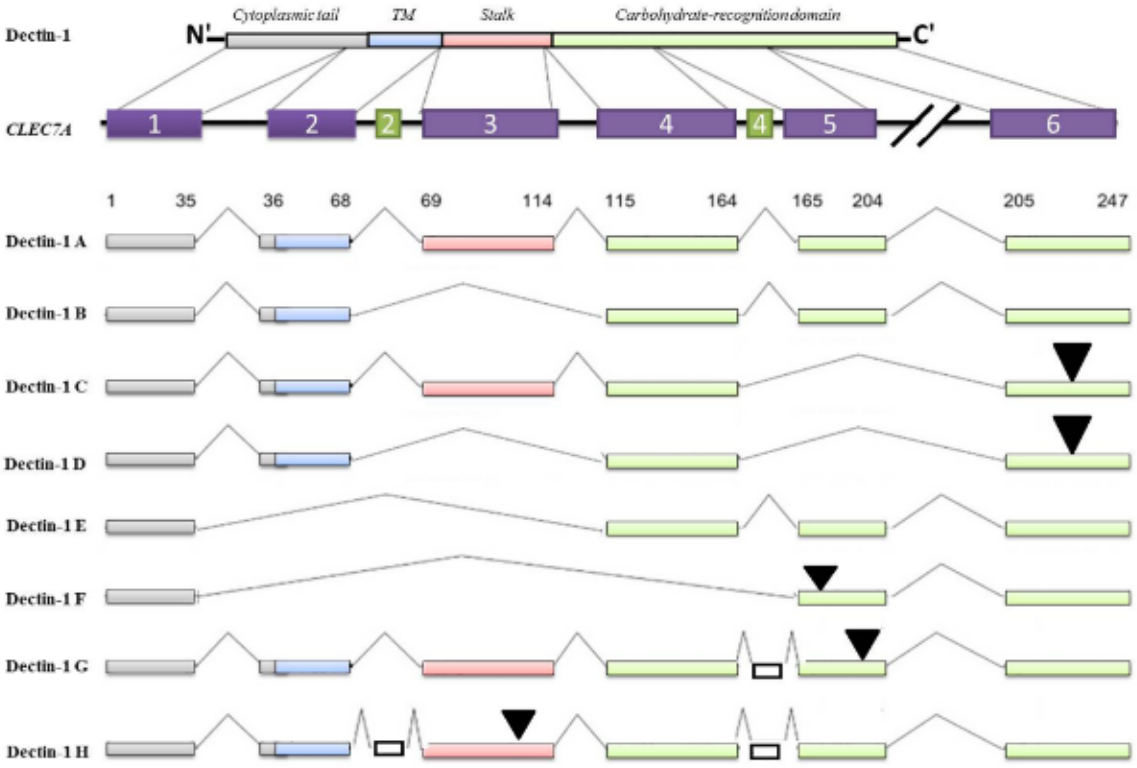

Figure 3: Representation of several spliced Dectin-1 isoforms. The location of the stop codons, which are produced via alternative splicing, are indicated by the inverted black funnels. Different Dectin-1 isoforms were produced as a result of exon loss, with the exception of Dectin-1 G and H. Both of them were produced as a result of the insertion of additional short exon into intron 2 and/or 4. (Figure adapted from: Kalia, et al., 2021)

1.3.2 Signaling via Dectin-1

Activation of Dectin-1 receptors in response to fungal infection can trigger a range of downstream cellular responses (Fig. 4), including the production of cytokines such as TNF-a, IL-1b, IL-2, IL-8, IL-10, IL-12, and CXCL2, induction of phagocytosis, and generation of reactive oxygen species through respiratory burst. In addition to these innate immune responses, Dectin-1 signaling can also drive the differentiation of CD4+ T cells to a Th1 or Th17 phenotype and activate CD8+ T cells to promote adaptive immunity. Dectin-1 is the main receptor on myeloid cells for beta 1,3-linked glucans, carbohydrates that are present in certain bacteria as well as the cell walls of plants,

fungi, and other organisms (G. D. Brown et al., 2003; Goodridge et al., 2007; Mata-Martínez et al., 2022; Underhill et al., 2005a).

Traditional immunoreceptor tyrosine-based activation motif (ITAM) sequences are tyrosine phosphorylated by Src family kinases upon ligand binding in myeloid cells, supplying recognition sites for Syk, which then starts all subsequent downstream signaling events (Ivashkiv, 2009; Lowell, 2011).

Dectin-1, like the other activation receptors, undergoes ligand-induced tyrosine phosphorylation by Src kinases, which causes Syk to be recruited (Gantner et al., 2003). Nevertheless, the signaling pathways activated downstream of Dectin-1 can be subdivided based on their reliance on Syk. The Syk dependent pathways include: Phosphatidylinositol 3-Kinase (PI3K)/AKT pathway, Phospholipase C-g2 (PLC-g2) and Ca2+- Dependent Activation, CARD9/BCL10/MALT1 Complex-Mediated Signaling and the Dectin-1/Syk/CARD9/IRF pathway (Mata-Martínez et al., 2022). The Syk independent pathways relies on the Raf-1 kinase (also known as c-Raf) and PI3K/AKT, leading to phagocytosis and the production of cytokines (Kalia et al., 2021).

In order to connect Dectin-1/Syk activation to Bcl10-Malt1-dependent NF-kB activation for innate anti-fungal immunity, Card9 functions as a critical adapter for non-TLR PRR signal transduction (Gross et al., 2006). The study carried out by Gross et al proved that mice deficient in CARD9, BCL-10 or MALT1, exhibit defective Dectin 1-induced NF-κB activation, and the survival of CARD9-deficient mice following infection with C. albicans is impaired. The formation of this complex is crucial for activating NFkB which in turn promotes the production of inflammatory cytokines like pro-IL-1b, IL-6, and TNF-a. Moreover, it has been shown that Dectin-1 together with TLR2 (Toll like receptor 2) works together and stimulates the antifungal response of the host cell (Dillon et al., 2006). Additionally, according to immunofluorescence experiments, both receptors colocalize on phagosome membranes (Inoue & Shinohara, 2014; Shin et al., 2008). Experiments on coimmunoprecipitation have suggested that Dectin-1 and TLR2 interact physically (Shin et al., 2008). Yet, additional investigations have demonstrated that rather than a direct contact between the 2 receptors, the synergistic

activity of the 2 receptors is caused via common signaling pathways (Gantner et al., 2003).

A recent study, carried out by Li et al, demonstrated that the immunological responses primarily induced by TLR2 activation, such as cytokine release, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-B), and reactive oxygen species (ROS) generation, are enhanced by Dectin-1. However, the nanoscale closeness of the receptor pairs is necessary for this signaling synergy (Li et al., 2019). Moreover, this demonstrated that Dectin-1 and TLR2 must be in close contact (nanoscale) for their complementary immunological roles. Another important key finding in this study was that Dectin-1 and TLR2 during signaling do not colocalize but instead form distinct clusters, which is in contrast to earlier studies (G. D. Brown et al., 2003; Li et al., 2019; Shin et al., 2008).

Dectin-1 contains a unique "hemi-ITAM" motif, which has a single YXXL sequence instead of the typical dual YXXL found in conventional ITAMs (Kerrigan & Brown, 2010).. Despite this difference, Dectin-1 can still activate downstream signaling in myeloid cells, such as macrophages, dendritic cells, and neutrophils, when it binds to β-glucan-containing particles like zymosan and curdlan. This activation triggers Src/Syk-dependent signaling pathways, which activate mitogen-activated protein (MAP) kinases and transcription factors such as NF-kB and NFAT (Goodridge et al., 2009).

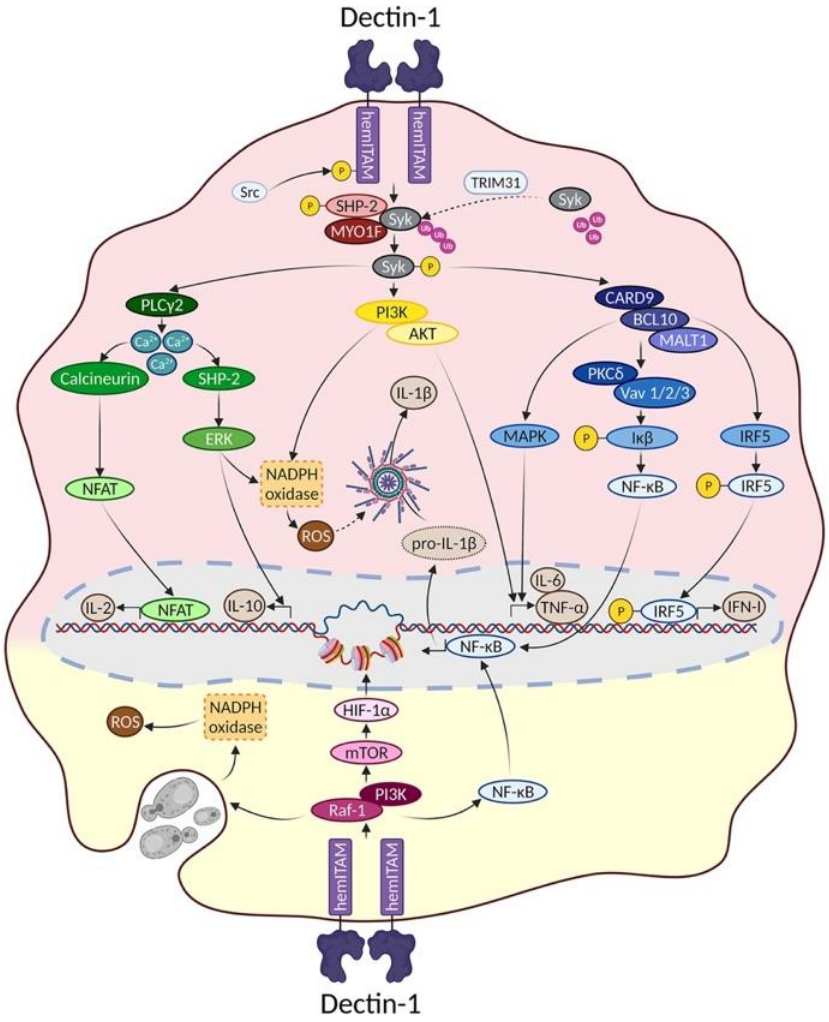

Figure 4: Representation of Dectin-1 signaling in immune cells. Dectin-1 with its hemi-ITAM motifs gets activated upon binding to glucans, with the phosphorylation of Syk being its consequence. Dectin-1 can activate several pathways including NFAT and NFkB.
(Figure adapted from: Mata-Martinez et al., 2022)

1.4 Glucans

Glucan is not to be confused with glycan. The International Union of Pure and Applied Chemistry (IUPAC) has defined the terms glycans and polysaccharides as being synonymous, with both terms referring to “compounds that comprise a large number of monosaccharides that are linked together glycosidically” (Chemistry (IUPAC), n.d.). Glycans, also known as carbohydrates, are just as important to the field of biology as nucleic acids and proteins. Many pathways in biology involve protein glycosylation,

which can occur in several different forms including N-linked glycosylation, mucin-type glycans, GlcNAc, fucose, mannose, and glucose. These post-translational modifications play a crucial role in a wide range of cellular and extracellular functions, and have a significant impact on these processes (van Kooyk & Rabinovich, 2008; J. Y. Zhou et al., 2018).

Glucans, however, are a type of polysaccharide that consists of D-glucose molecules linked together by glycosidic bonds (Kamoun et al., 2017). Glucans are classified based on their type of linkage and the carbons involved in the binding. There are two main types of glucans: alpha-linked and beta-linked. These two classes have different characteristics and functions. Alpha glucans are typically amorphous and soluble in hot water and serve as an energy reserve material. Examples of alpha glucans include glycogen in fungi and animals, and starch in algae and plants. However, the alpha glucans present in fungal walls are water-insoluble and microfibrillar, meaning that they are formed by the association of several glucan chains through hydrogen bonding and serve mostly a structural role. Most beta glucans are insoluble in water and most solvents and are crystalline in structure. They can also form microfibrils, similar to those formed by alpha glucans, through the association of multiple chains (Ruiz-Herrera & Ortiz-Castellanos, 2019).

Glucans have been shown to have a role in immune signaling. The beta-glucans found in the cell walls of fungi have been reported to activate the immune system by binding to specific receptors on immune cells such as macrophages, neutrophils and natural killer cells (Goodridge et al., 2009).

1.5 Beta glucans

Beta glucans are polysaccharides found in the cell walls of bacteria, fungi, and grains, composed of glucose units linked by β-glycosidic bonds. Their structure varies depending on the source organism. Typically, they have a linear backbone with β-1,3 bonds, and yeast and fungal β-glucans also have β-1,6 side branches, while cereal β-glucans contain both β-1,3 and β-1,4 backbone bonds (Bin Du et al., 2019) (José Ruiz-Herrera & Lucila Ortiz-Castellanos, 2010; Zeković et al., 2005).

Linear beta-D-glucans have been identified in many fungal sources, including lichens. These polysaccharides are composed of either (1→3)-linked or (1→6)-linked -D-Glcp units. While cellulose-like (1→4)-linked-D-glucans have not been found in fungal sources, some lichenized fungi have been found to have linear mixed linkage -D-glucans (lichenans) that combine both (1→4) and (1→3) linkages (Synytsya & Novák, 2013).

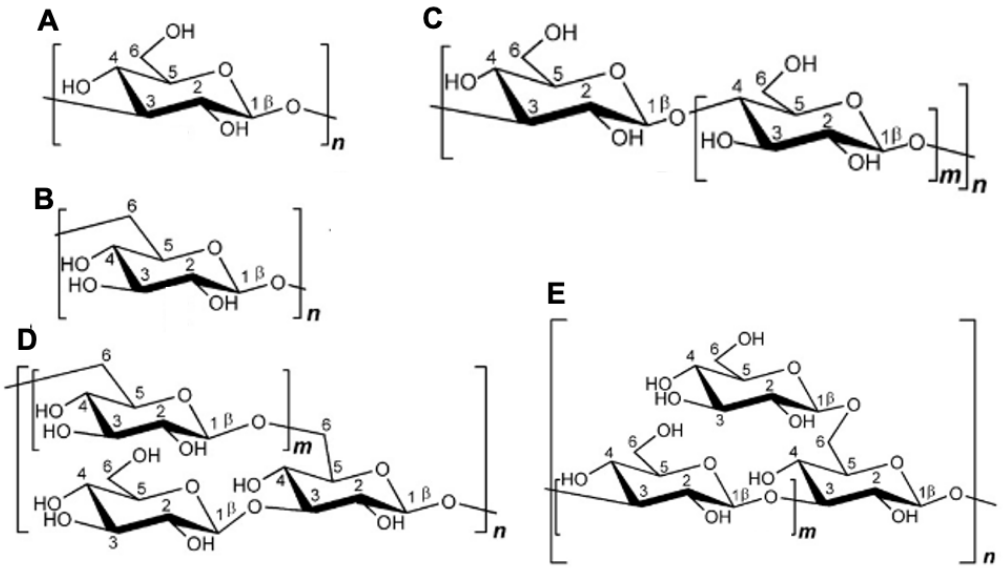

Figure 5: Different beta-glucan forms. (a) (1→3)-beta-D-glucan, (b) (1→6)-beta-D-glucan, (c) mixed linkage (1→3),(1→4)-beta-D-glucan, (d) branched (1→3),(1→6)-beta-D-glucan, (e) branched (1→6),(1→3)-beta-D-glucan (Figure adapted from: Synytsya & Novák, 2013)

Beta-glucans are found to have a positive impact on cancer and infection diseases. Dectin-1 can recognize fungal beta-glucans and thus stimulate the canonical and non-canonical immune signaling pathways (Camilli et al., 2018). Studies have also shown that beta-glucans can enhance the immune response to vaccines and reduce the risk of infections. Additionally, beta-glucans have anti-inflammatory properties and have been studied for their potential in treating various inflammatory conditions (Żyła et al., 2021).

Research has shown that β-glucan-induced training affects inflammation-related genes at both genetic and epigenetic levels. β-glucans create a memory in innate immune cells by altering the methylation and acetylation of histone promoters and enhancers. This occurs during the first exposure to β-glucans, initiating gene transcription and leaving distinct chromatin marks that may persist even after the initial stimulus is removed.(Camilli et al., 2018; S.-C. Cheng et al., 2014; Saeed et al., 2014).

1.5.1 Dectin-1 signaling upon beta-glucan infection

Dectin-1 serves as the key receptor for the phagocytosis of fungal particles, particularly those with β-glucan-rich cell walls, such as yeast (Brown et al., 2002). While TLRs detect fungal components and trigger the production of pro-inflammatory cytokines, they do not directly mediate the internalization of these particles (Underhill et al., 1999). Studies have demonstrated that Dectin-1 enables both non-phagocytic and immune cells, including macrophages and dendritic cells, to efficiently bind and ingest β-glucan particles. This receptor is especially effective in the phagocytosis of zymosan and live fungi, playing a crucial role in the immune response to fungal infections (Brown et al., 2002; Saijo et al., 2007; Taylor et al., 2007).

Moreover, Dectin-1, in contrast to other PRRs, discriminates between soluble and particulate β-glucans. A study by Goodrige et al. tried to clarify how Dectin-1 distinguishes between direct microbial contact and detection of soluble glycans. The study showed that Dectin-1 signaling specifically responds to particulate β-glucans by forming synapse-like structures that exclude regulatory tyrosine phosphatases CD45 and CD148, activating the MAPK/NFkB signaling pathways. Dectin-1 plays a key role in antifungal defense, triggering distinct antimicrobial responses through Src and Syk kinases, and influencing both innate and adaptive immunity. (Goodridge et al., 2011).

The paper compared the effects of particulate and soluble β-glucans on antimicrobial and inflammatory responses, showing that soluble β-glucans need to be immobilized to activate Dectin-1 signaling. Although soluble β-glucans bind efficiently to Dectin-1, they fail to activate the receptor. The study also found that CD45 and CD148 regulate Dectin-1 signaling in macrophages by being excluded from the contact site during

phagosome formation. This highlights the importance of phagocytic synapses in Dectin-1 activation and proposes a mechanism through which Dectin-1 distinguishes between soluble and particulate ligands, leading to hemi-ITAM phosphorylation and Syk kinase recruitment. (Goodridge et al., 2011).

Dectin-1-mediated phagocytosis is dependent on ITAM phosphorylation and the activation of Src family kinases. The phagocytosis of β-glucan-containing particles is not impaired by the inactivation of Syk in genetically or pharmacologically inactivated macrophages. The translocation of Dectin-1 to phagosomes containing β-glucans and the following activation of Syk allow for the maturation and acidification of phagosomes containing C. albicans and β-glucans in macrophages (Mansour et al., 2013). It is thus important for β-glucan binding to Dectin-1 to not only trigger phagocytosis, but also allow for the lysosomal fusion and acidification of the phagocytic compartment (Goodridge et al., 2009; Underhill et al., 2005b).

1.6 Langerin

Another type II C-type lectin receptor is called langerin, also known as CD207 (Thépaut et al., 2009; Valladeau et al., 2000). It is involved in the attachment and absorption of pathogens during the early phases of the immune response. It is exclusively present on the surface of Langerhans cells, a type of immune cell found in the skin and mucous barrier. As all C-type lectin receptors, Langerin as well is involved in the immune system's response to invading pathogens, including Candida albicans and human immunodeficiency virus (HIV) (de Witte et al., 2007) and has been shown to play a role in the development of autoimmune diseases such as type 1 diabetes (Doss & Smith, 2012). It can recognize mannose, fucose and *N*-acetylglucosamine structures in a Ca^{2+} dependent manner.

1.6.1 Structure

Like other type 2 transmembrane C-type lectin receptors, Langerin has an extracellular region consisting of a neck and a single C-terminal C-type carbohydrate recognition

domain (CRD). As an oligomer, Langerin forms trimers that are stabilized by a coiled coil of alpha - helices in the neck area. Because the CRD of Langerin has only a modest affinity for monosaccharides, as is common for C-type CRDs, trimer formation is necessary for binding to oligo-saccharide ligands. The CRD also includes an EPN (glutamate-proline-asparagine) motif, indicating an affinity for mannose (which allows the recognition of viruses, fungi and mycobacteria (Geijtenbeek & Gringhuis, 2009). There are two potential N-glycosylation sites located at amino acid positions 87–89 and 180–182. Additionally, Langerin has an intracellular domain consisting of 43 amino acids, featuring a proline-rich motif (WPREPPP) which possibly serves as a signal transduction site. Especially proline-rich motifs can serve as a recognition site for SH3 domains (Src Homology 3 domains). The primary function of those domains is to facilitate protein-protein interaction via sequences that contain proline and hydrophobic amino acids. The interaction between those domains and Langerins proline-rich domain is crucial for regulating the dynamics of the signaling transduction (regulating vesicle trafficking and cytoskeletal movement) within the cell (Cohen et al., 1995; Valladeau et al., 2000, 2002).

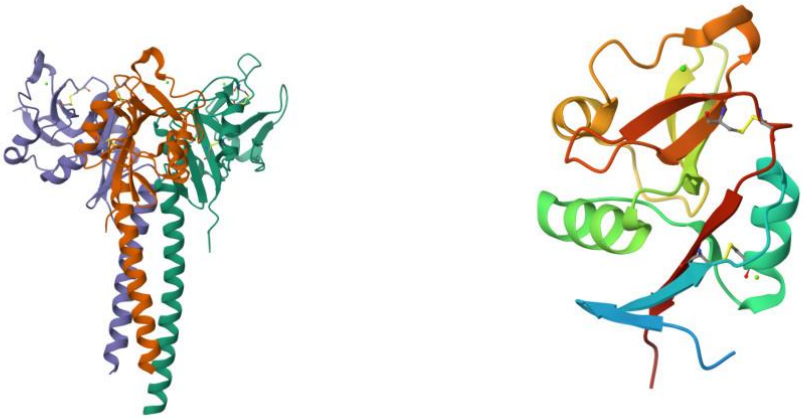

Figure 6: Structure of human Langerin. Langerin is structured with a concise intracellular segment and an extracellular domain composed of a neck region and a carbohydrate recognition domain (CRD) (right image). Within its intracellular portion lies a proline-rich domain (PRD). The neck region is characterized by alpha-helices, facilitating the assembly of langerin homotrimers through coiled-coil interactions. These homotrimer formations enhance both the avidity and specificity of antigen binding. The structure of langerin, including its extracellular domain, transmembrane region, and cytoplasmic tail, enables the receptor to bind and internalize pathogens, leading to immune cell activation and the induction of adaptive immune responses. (https://doi.org/10.2210/pdb3C22/pdb)

1.6.2 Langerin Signaling

After recognition and binding of specific carbohydrate structures on the pathogens surface, Langerin helps to internalize the pathogen. It transports it to a specialized compartment within the Langerhans cells which are called the Birbeck granule. Up to date, the exact function and shape of Birbeck granules remains unknown. Initial studies have suggested that Birbeck granules are supposed to function as alternative endocytic structures, which is specific to Langerhans cells (Takigawa et al., 1985). However, a study carried out by McDermott et al contradicts this hypothesis, as they found out that Langerin endocytosis is mediated by a clathrin-coated pathway rather than Birbeck granules (BG), hence BGs are not endocytic structures (Mc Dermott et al., 2002).

Although for a long time it has been thought that only Dectin-1 is able to recognize and bind to β-glucans, studies have shown that h-Langerin as well has the capability. The study carried out by de Jong and coworkers found out that Langerin exhibited strong interactions with curdlan, laminarin, and zymosan. Curdlan and laminarin are rich in β-glucans, while zymosan, derived from S. cerevisiae, contains both mannose and β-glucans. This indicates that Langerin as well can interact with a variety of Candida species and is most responsible for most Candida species interactions. All of these findings indicate that Langerin and DC-SIGN and dectin-1 have similar carbohydrate specificities, which was not known before (de Jong et al., 2010; Hanske et al., 2017). Crystallographic studies showed that a calcium cation might be coordinated by the non-reducing glucose residue via hydrogen bonds with its OH-3 and OH-4 functional groups (De Jesus et al., 2014). It is yet unclear what the direct downstream consequences of receptor activation are.

After identifying and internalizing pathogens through endocytosis, Langerin also has the ability to uptake and process antigens for presentation via both major histocompatibility class I and class II molecules, playing a crucial role in cross-presentation of antigens to T cells (Hunger et al., 2004).

In addition to its role in the immune system, Langerin has also been shown to play a role in the development of autoimmune diseases (Xuan et al., 2022). Additionally to that, Langerin has also been found to play a role in the development of cancer. In some types of cancer, Langerin expression has been found to be downregulated, leading to an impaired immune response and an increased risk of cancer progression (Zhou et al., 2022)

1.7 Immune Signaling Pathways

Two major downstream signaling pathways will be described in more detail, since they both play an important role in this thesis. Both pathways are central players in the host immune response to pathogens, since both ERK and NFkB pathways can induce induction of pro-inflammatory cytokines, chemokines, and both are activated by receptor-mediated signaling.

1.7.1 ERK Pathway

Extracellular signal-regulated kinase 1/2 (ERK) is part of the mitogen-activated protein kinase (MAPK) family (Keshet & Seger, 2010). They belong to the serine/threonine protein kinases and are located in the cytoplasm. After activation they move to the nucleus where they control gene expression as well as activity of transcription factors. In total there are four different isotypes of ERK (Plotnikov et al., 2011).

For the MAPK/ERK pathway at first the GTPase Ras is activated after specific stimuli at the plasma membrane. This activates Raf, which is a MAP3K (mitogen activated kinase kinase kinase), including dimerization and phosphorylation. Afterwards, the signal is transmitted to MAP kinase kinases (MAPKKs), MEK1 and MEK2. They transmit their signal then to ERK1/2 (MAPKs) (Plotnikov et al., 2011). The signals then go to numerous additional substrates, which are found in the cytoplasm, nucleus, or other cellular organelles, as well as the MAPKAPK components (RSKs, MNKs, and MSKs) (Yoon & Seger, 2006). When dysregulated this pathway plays a huge role in cancer as well (Dhillon et al., 2007)

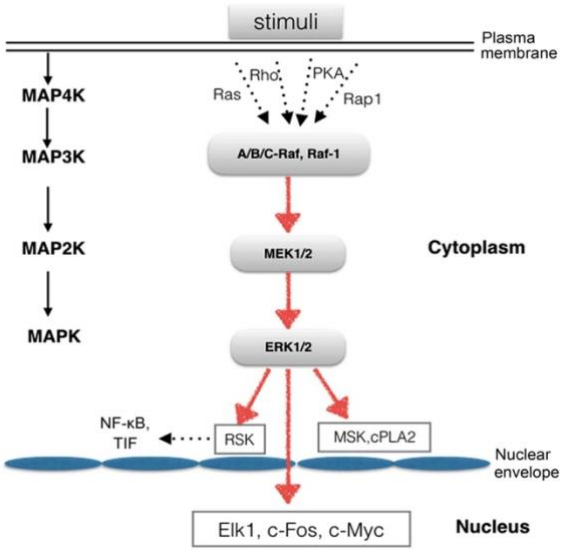

Figure 7: Schematic representation of the ERK pathway. This scheme represents the ERK pathway, from the stimulus until the production of transcription factors. The stimuli (binding of mitogen to cell-surface receptor) leads to the subsequent activation of Ras, which in further notice activates MAPKKs downstream. This pathway plays an essential role in regulating cell proliferation and growth. (Figure adapted from: P J Roberts & C J Der 2007)

1.7.2 NFkB pathway

The transcription factor family of Nuclear factor-κB (NF-κB) can be activated in response to various stimuli and plays a key role in regulating the expression of genes involved in immune and inflammatory responses across a range of physiological processes. It consists of five members: p50, p52, p65 (RelA), RelB and c-Rel. Without a signal, NFkB is usually sequestered in the cytoplasm by inhibitory proteins. IkB family is one example. Activation of NFkB is a result of the inactivation of IkB. The activation of a kinase called the IκB kinase (IKK) is the primary mechanism by which this process occurs. IKK is made up of a catalytic subunits IKKα and IKKβ, as well as a regulatory protein known as NEMO or IKKγ. Signals from outside the cell activate the IκB kinase, which then phosphorylates serine residues in an IκB regulatory domain. This phosphorylation leads to the ubiquitination and subsequent degradation of IκB proteins by the proteasome (Karin & Delhase, 2000; Oeckinghaus & Ghosh, 2009).

Nevertheless, there are two major pathways for the NFkB activation: the canonical and non-canonical one. While the canonical pathway relies on the degradation of IkB via IKK as mentioned above, the noncanonical NF-κB activation does not involve IκBα degradation but relies on processing of the NF-κB2 precursor protein, p100. Another

difference is the respond to stimuli. The canonical usually responds to diverse stimuli, including receptors of PRR, TCR and BCR. The non-canonical, however, is more specific and only responds to a very specific group of stimuli (Liu et al., 2017; Sun, 2011).

NF-κB has a variety of functions in the immune system and beyond. It is involved in regulating the expression of genes that are responsible for immune responses, such as cytokines and chemokines, as well as genes involved in cell survival, proliferation, and differentiation. In addition, NF-κB plays a role in inflammation, cell growth and survival, and regulating the response to stress. Moreover, NF-κB has been shown to play a complex role in cancer, with both oncogenic and tumor suppressive effects. On the one hand, NF-κB activation can promote cell survival and proliferation, as well as immune evasion and angiogenesis in tumor cells. On the other hand, NF-κB can also induce cell death and inhibit tumor growth by promoting immune responses and activating anti-tumor pathways (Tak & Firestein, 2001).

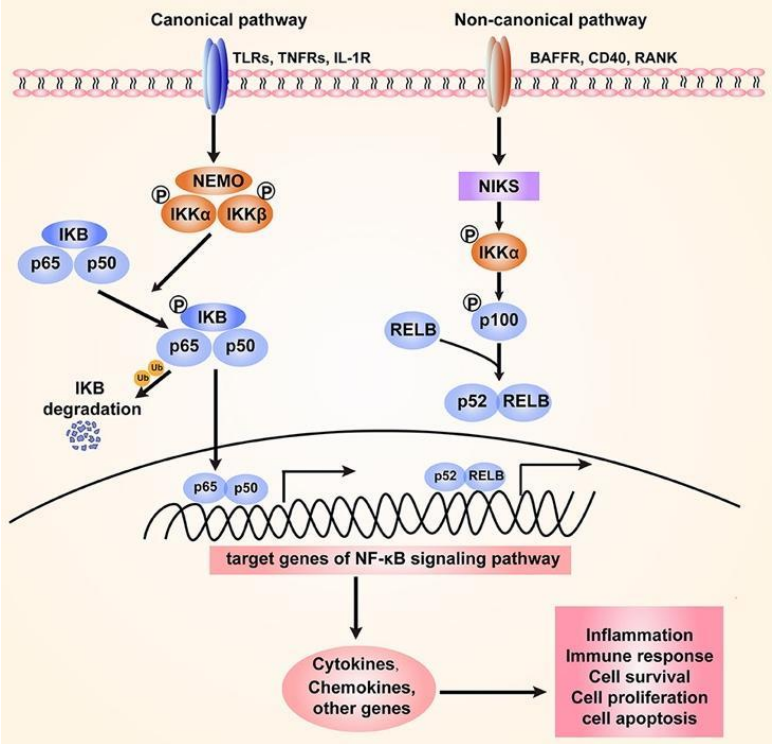

Figure 8: Schematic representation of the canonical and non-canonical NFkB pathway. The canonical NF-κB pathway is activated by extracellular stimuli such as proinflammatory cytokines (e.g., TNF-α, IL-1) or microbial products (e.g., LPS), which bind to cell surface receptors, leading to the activation of the IKK complex. This results in the phosphorylation and degradation of IκB proteins, freeing NF-κB dimers to translocate into the nucleus and regulate gene expression. On the other hand, the non-canonical NF-κB pathway is triggered by a subset of receptors from the TNF receptor superfamily (e.g., CD40, LTβR), which induce the stabilization and accumulation of NF-κB-inducing kinase (NIK). NIK activates IKKα homodimers, leading to the processing of p100 to p52 and the formation of active p52-RelB dimers that translocate to the nucleus for transcriptional regulation. (Figure adapted from: Peng et al., 2020)

1.8 Fungi

1.8.1 Characteristics

Fungi belong to the eukaryotes and include yeasts, molds, and mushrooms. They share some features with the eukaryotes. Fungal cells, like other eukaryotes, have membrane-bound nuclei with chromosomes containing both coding (exons) and noncoding (introns) regions of DNA. They possess membrane-bound organelles such as mitochondria, sterol-containing membranes, and 80S ribosomes. Fungi reproduce both sexually and asexually, producing spores that can spread through the air, water,

or via animals. Many fungi are beneficial, such as *Saccharomyces cerevisiae* and *Penicillium* species. However, some can be harmful, causing diseases in plants or humans. Their characteristic feature is their cell walls since they are made of chitin, which distinguishes them from plants, which have cellulose-based walls (Deacon, 2013; Jennings & Lysek, 1999).

Like animals, fungi are heterotrophs, relying on complex organic compounds for carbon rather than fixing carbon dioxide like plants. They also cannot fix nitrogen and must obtain it from their diet. Unlike animals, fungi digest food externally by releasing exoenzymes from their hyphae to break down nutrients, which are then absorbed through their large mycelium surface. Fungi store polysaccharides as glycogen, similar to animals. Most fungi are saprotrophs, obtaining nutrients from decaying organic matter, primarily plant material. Many fungi produce biologically active compounds, some of which are toxic to animals or plants and are known as mycotoxins (McGinnis & Tyring, 1996) .

1.8.2 Cell wall

The fungal cell wall mediates interactions with the external environment. It is mostly composed of polysaccharides, including chitin, β-glucans, and mannans, along with proteins, lipids, and pigments. The cell wall's primary structural components are chitin and β-glucans. Chitin, a polymer of N-acetylglucosamine, is needed for the strength of the cell wall. Disruptions in chitin synthesis or structure can compromise cell wall integrity, potentially leading to cell lysis and death. β-glucans, including β-1,3-glucan and β-1,6-glucan, contribute to the cell wall's structural framework and have been implicated in various cellular processes, including cell wall remodeling and defense. The fungal cell wall also plays a critical role in interactions with the external environment. Certain cell wall components, such as β-glucans and mannans, are highly immunogenic and can elicit both cellular and humoral immune responses during fungal infections (Fontaine et al., 1997; Hasim & Coleman, 2019; Kapteyn et al., 1999).

1.9 Bacteria

1.9.1 Characteristics

Bacteria (formerly Eubacteria) belong to the domain prokaryotes, alongside Archaea, therefore, only consist of one single cell that lacks a nucleus and other cell organelles (endoplasmatic reticulum, mitochondria, etc). They are ubiquitous meaning they are found in virtually every ecological niche on earth (Susan Sherwood, 2019). Bacteria have a single circular chromosome and multiple plasmids. Plasmids are small, circular, double-stranded DNA structures that naturally occur in all bacterial cells and play roles in replication, food digestion, and other cellular processes. Bacteria can reproduce through both sexual and asexual methods, but the majority rely on binary fission as their primary mode of reproduction. The classification of bacteria is based on their mode of respiration (aerobic, anaerobic), shape (see below), nutritional pattern (autotrophic, heterotrophic), reproduction (binary fission, conjugation, transduction and transformation) and cell wall composition (gram-positive, gram-negative) (Robert J. Kadner & Kara Rogers, 2024).

Nearly all areas of the world are home to bacteria, which are essential to its ecosystems. Several species can survive in environments with high pressure and temperatures. Often times, without our knowledge, humans and microbes coexist in a symbiotic interaction. (*What Are Bacteria?* | *Live Science*, n.d.).

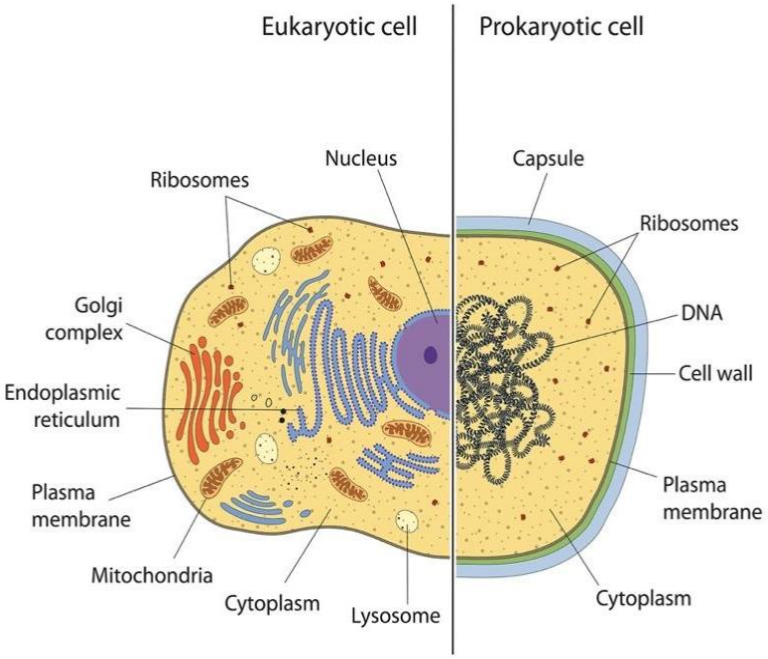

Figure 9: Comparison between eukaryotic and prokaryotic cell. While the eukaryotic cell consists of several membrane-bound organelles, prokaryotes do not contain such things. Moreover, prokaryotic cells do not have a nucleus. Eukaryotic DNA is housed in the nucleus, in contrast to prokaryotic DNA; which resides in the cytoplasm. Furthermore, eukaryotic DNA is structures as double-stranded chromosomes compacted by histones, while prokaryotes have a single circular chromosome and multiple plasmids, which are small circular pieces of DNA. (Figure adapted from: Latham, 2021)

1.9.2 Cell wall structure

The cell wall of bacteria is necessary to protect the bacteria from the internal osmotic pressure. This is brought on by the presence of significantly higher concentrations of proteins and other molecules inside the cell than in the surrounding environment. Basically, it is an essential component in order to maintain its structural integrity (Bruslind, 2019).

Peptidoglycan is an essential part of the cell wall. Linear glycan strands linked together by short peptide sequences make up the primary structural components of peptidoglycan. The N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues that make up the glycan strands are alternated and connected by b-1 → 4 linkages (Vollmer et al., 2008). Although some structural features of the peptidoglycan are conserved throughout all bacterial species, some variations in, for

example in the peptide stem or glycan strand, do exist. Usually it is between species, however, minor variations can also occur within the same species, depending on the growth conditions (Schleifer & Kandler, 1972).

Two main types of cell walls can be distinguished: gram-positive and gram-negative bacterial cell wall.

Gram positive cell wall

The major difference between the gram-positive and gram-negative cell wall layer is the thickness of peptidoglycan layer. The layer of peptidoglycan for gram-positive bacteria can have a size of up to 100nm, making them visible in light microscopy. Several models for the gram-positive cell wall have been suggested over the past years, including layered and scaffold model.

Moreover, they lack the outer membrane (in contrast to gram-negative bacteria). Instead, they have teichoic acid (first described in 1959), which can be divided into lipoteichoic acid (LTA) and wall teichoic acid (WTA) (Armstrong et al., 1959). In general, teichoic acids are copolymers of glycerol phosphate or ribitol phosphate and carbohydrates linked via phosphodiester bonds and are highly negatively charged (Ward, 1981).

While LTA is retained on the cytoplasmic membrane by a lipid anchor, WTA are covalently connected to the peptidoglycan. A disaccharide linkage unit (highly conserved) and a main chain polymer made up of phosphodiester-linked polyol repeat units make up the wall teichoic acid polymer (Neuhaus & Baddiley, 2003). N-acetylmannosamine (beta 1- 4) N-acetylglucosamine-1-phosphate (ManNAc (beta 1-4) GlcNAc-1P) and one to two glycerol-3-phosphate (GroP) units connected to the C4 oxygen of ManNAc make up the disaccharide linkage unit (Araki & Ito, 1989). This linkage unit is common for all WTAs in bacteria; however, variations exist in their repeat units. The phosphodiester-linked polyol repeat units of 1,5-d-ribitol-phosphate (RboP) or 1,3-l-α-glycerol-phosphate (GroP) belong to the best-characterized WTA structures. Contrarily, LTA structure consists typically of a polyglycerolphosphate

(PGP) chain connected to the bacterial membrane by a glycolipid anchor (Reichmann & Gründling, 2011).

Gram negative cell wall

The cell wall of gram-negative bacteria is unique and more complex than that of gram-positive bacteria. It is composed of a thin layer of peptidoglycan surrounded by an outer membrane made up of lipopolysaccharides (LPS), protein, and phospholipids. The outer membrane (OM) is a lipid bilayer, like other biological membranes, but it is distinct in that it is not a phospholipid bilayer. While phospholipids are present in the OM, they are restricted to the inner leaflet of the membrane. The LPS layer is what gives gram-negative bacteria their characteristic staining pattern, and also plays a role in pathogenesis and resistance to antibiotics. Additionally, gram-negative bacteria have specialized structures called porins that allow for the transport of nutrients and other molecules across the outer membrane (Kamio & Nikaido, 1976; Silhavy et al., 2010). LPS, the main component of the outer membrane, consists of three parts: the lipid A, core oligosaccharide, and O-antigen. LPS is responsible for many of the pathogenic properties of Gram-negative bacteria, such as endotoxic activity, and can activate the host immune system (Matsuura, 2013).

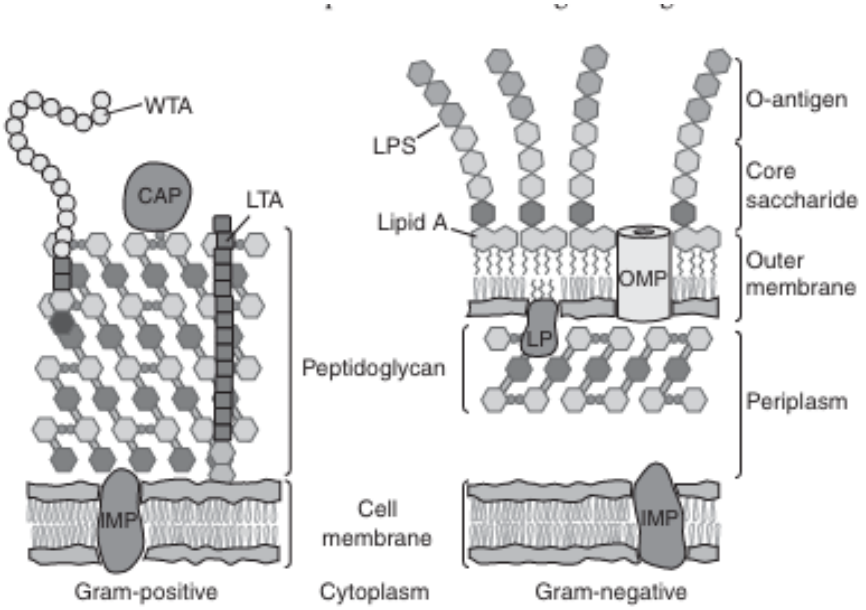

Figure 10: Difference between gram-positive and gram-negative cell wall. The gram-negative and gram-positive cell walls of bacteria differ in a number of ways. Gram-positive cell walls of bacteria are thick and composed primarily of peptidoglycan, with teichoic acid and lipoteichoic acid further contributing to the rigidity of the cell wall. In contrast, gram-negative cell walls are thinner and composed of a mixture of peptidoglycan, lipoproteins, and lipopolysaccharides, which form the outer membrane layer. The lipopolysaccharides of the outer membrane also contribute to the pathogenicity of gram-negative bacteria by acting as endotoxins. Additionally, gram-negative bacteria have a periplasmic space between the outer and inner membranes that contains enzymes involved in cellular metabolism. (Figure adapted from: Kamio & Nikaido, 1976)

Two gram-positive bacteria will be described in further detail.

1.10 Staphylococcus aureus

1.10.1 Characteristics

Staphylococcus aureus is a gram-positive bacterium with a spherical shape (cocci). It belongs to the family Staphylococcaceae, which belongs to the phylum Bacilotta. Moreover, it is facultative anaerobic, non-motile, catalase-positive and it reproduces asexually (Canada, 2012). It can be found in the microbiome of the human gut (commensalism). However, it can become a pathogen being the cause of skin, bone, as well as bloodstream infections (S. aureus is the leading cause of bloodstream infections) (Tong et al., 2015).

The cell wall is common for all gram-positive bacteria, with peptidoglycan being its major component, forming the sacculus. The glycan chains, consisting of MurNAc and GlcNAc are joined by short peptides that are peptides attached to the MurNAc residues. L-alanine, D-isoglutamine, L-lysine with a penta-glycine connected to the epsilon amino group, and a terminal D-alanine-d-alanine make up the nascent stem peptides in S. aureus PG (Vollmer et al., 2008). Moreover, WTA is highly abundant as well in the cell wall. Most S. aureus variants synthesize RboP-WTA.

In this master thesis, three WTA glycosyltransferases which are essential for S. aureus are of importance and will be discussed further.

WTA glycosyltransferase - tarM

TarM was the first enzyme to be discovered and is a soluble cytoplasmic glycosyltransferase (GT) enzyme. The Rbo-P-type WTA is initially characterized as being glycosylated by TarM, and TarM is also the first WTA glycosyltransferase whose activity has been demonstrated in vitro using a recombinant method. It catalyzes an *O*-linked α- and β-GlcNAc glycolysation at the C4 position of Rbo-P (Xia et al., 2010).

WTA glycosyltransferase - tarS

TarS, also known as housekeeping WTA glycosyltransferase is highly conserved in S.aureus genome. It is responsible for β-O-GlcNAcylation of Rbo-P at C4. It uses the same substrates as tarM (UDP-GlcNAc and RboP-WTA), but with a different linkage (Solmaz Sobhanifar et al., 2016).
Interestingly, β-O-GlcNAcylation of *S*. *aureus* WTA is specifically responsible for methicillin resistance in MRSA (methillicin resistant staphylococcus aureus). Deleting tarS results in a resensitization of MRSA strains to beta-lactam antibiotics (S. Brown et al., 2012).

WTA glycosyltransferase – tarp

TarP was found most recently as another important glycosyltransferase. Instead of catalyzing the attachment of GlcNAc to the poly-RboP backbone via a b-O-linkage at C4 position, it modifies the C3 hydroxyl group of RboP (Gerlach et al., 2018). Furthermore, TarP-mediated C3-b-O-GlcNAcylation of WTA can confer b-lactam resistance to S. aureus (Guo et al., 2021).

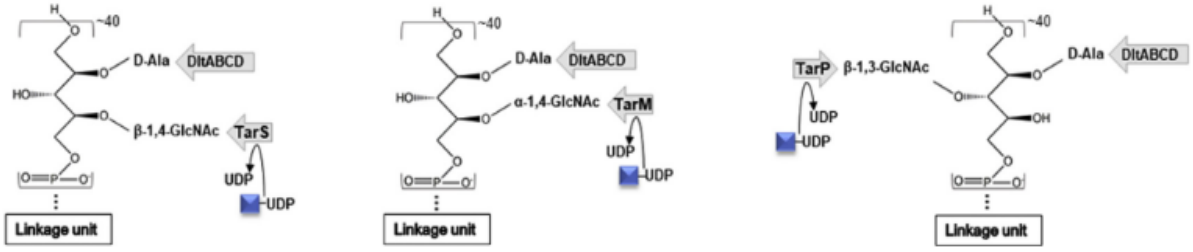

Figure 11: Representation of the different proteins tarS, tarM and tarP. TarM is a transferase that adds an O-linked N-acetylglucosamine (O-GlcNAc) modification to the wall teichoic acid (WTA) of S. aureus, while TarP and TarS are glycosyltransferases that are involved in adding the sugar residues galactose and N-acetyl-galactosamine (GalNAc), respectively, to the WTA. TarP is also believed to play a role in biofilm formation. Overall, these proteins contribute to the virulence of S. aureus by modifying the WTA, a key component of the bacterial cell wall. (Figure adapted from: Guo et al., 2021)

1.11 Streptococcus pyogenes

1.11.1 Characteristics

Streptococcus pyogenes are gram-positive, non-motile, facultatively anaerobic and extracellular bacteria. Moreover, they are beta-hemolytic (when grown on blood agar, they typically produce small (2–3 mm) zones of beta-hemolysis, a complete destruction of red blood cells). They belong to the genus Streptococcus which belong to the phylum Bacillota (Canada, 2001). It is a major human-specific pathogen, than is the cause of a wide range of clinical manifestations, that can range from mild infections to life-threatening invasive infections, like sepsis, necrotizing fasciitis and streptococcal toxic shock syndrome (STSS) (Ibrahim et al., 2016).

Streptococcus pyogenes are also referred to as group A streptococcus (GAS), since they have the Lancefield group A antigen. S. pyogenes has been found to have a number of virulence factors that are encoded by the genome, including pili, M proteins, leukocidins, streptolysins, complement-inhibiting proteins, immunoglobulin-degrading enzymes, and superantigens (Walker et al., 2014).

Virulence factor - M protein

The M protein is a major surface protein and virulence factor of GAS, which is anchored to the cell wall peptidoglycan (Barnett & Scott, 2002). M proteins contain a motif at their C-terminus within the cell wall that is now recognized as being shared by a large number of Gram-positive bacterial surface proteins. LPXTGE, a conserved hexapeptide, is part of the motif (V. a Fischetti et al., 1990). It is strongly anti-phagocytic by binding to serum factor H (complement control protein, with its main function being regulating the alternative complement pathway (Hakobyan et al., 2008), destroying C3-convertase (belongs to serine proteases and is needed for the complement system (Craig A. Smith et al., 1984)) and preventing opsonization by C3b (V. A. Fischetti, 1989).

Virulence factor - gacI

S. pyogenes rely on several mechanisms to evade the hosts defense mechanism. A variety of virulence factors, including the Group A carbohydrate (GAC), are known to help these pathways. GAC is a polysaccharide and is proposed to be synthesized by 12 proteins, GacABCDEFGHIJKL, encoded in one gene cluster (Rush et al., 2017).
A polyrhamnose backbone and an immunodominant N-acetylglucosamine (GlcNAc) side chain make up the overall structure of Gac. The Gac makes up around 50% of the cell wall of GAS. Studies have discovered that especially the first 8 genes gacA-G and gacL are essential for the survival of the bacteria (Le Breton et al., 2017; van Sorge et al., 2014).

1.12 Host-pathogen interactions

Increasing evidence shows a strong link between post-translational modifications (PTMs) like glycosylation and bacterial virulence.

Glycosylated bacterial surface proteins not only act as adherence factors for bacterial attachment but also modulate host responses by directly modifying host proteins, without triggering the lectin response. Additionally, bacterial glycosyltransferases and glycosidases can alter host glycans as part of the pathogenic process. For example, bacteria produce surface structures, such as capsules, to inhibit host immune functions, and some mimic host glycan structures to evade host recognition. However, glycointeractions play a huge role in all stages during bacterial infection (De Oliveira et al., 2017; Lee et al., 2022; Poole et al., 2018).

Goal of this work

The goal of this work: i. is to examine further the glycan-receptor interaction of Dectin-1 and Langerin with soluble beta-glucans, added from solution or presented on yeast mimetic particles, or with the engineered bacterial strains and ii. to correlate such recognition with the activation of the downstream intracellular signaling. This will help solving the question how Dectin-1 and Langerin induce immune signaling pathways.

On today, there is no quantitative approach enabling to probe this sort of interaction at sufficient spatial and temporal resolution. This implies that we do not have a complete mechanistic understanding of how the spatial presentation of glycans affects the activation of cell signaling, in particular activation of Dectin-1 signaling. On the other hand, the effect Dectin-1/Langerin co-recognition of the same ligand has not been investigated in detail.

This work will try to explore intracellular communication through the glycans and can be applied further to investigate how the spatial presentation of glycans (nanoclustering) or how the physical properties of bacterial glycans (WTA, LTA; LPS, capsule polysaccharides) affect the activation of the innate immune response through the variety of lectin-receptors. Another goal of this work is to establish new experimental approaches to investigate glycan signaling at high-resolution, as new approaches are needed to access this information.

2. Methods

2.1 Cell culture

For this thesis U-937 suspension cell-line and HEK-293 T adherent cell-line were used. Suspension cells were cultured in RPMI 1640 (Gibco) with 10% FBS (fetal bovine serum) (Sigma-Aldrich), 1% Penicillin-Streptomycin (Gibco), and 1% Glutamax (Gibco). Adherent cells were cultured in DMEM with the same additions. All cells were cultured at 37°C with 5% CO_2.

U937 cells were kept between 1e6 and 2e6 cells/mL in full media with passaging 2-4 times a week. HEK293T cells were adherently cultured in DMEM with the supplements named above and split 2-3 times per week.

U-937

U-937 is a human myeloid leukemia cell line that was derived from the peripheral blood of a patient with diffuse histiocytic lymphoma. They grow in suspension, and they can be maintained in culture as a monolayer or in suspension culture. They are known to express a wide range of genes involved in myeloid differentiation, including CD14, CD11b, and CD11c. They also produce cytokines and chemokines, and respond to various stimuli such as lipopolysaccharide (LPS) and interferon-gamma (IFN-gamma) (Chanput et al., 2015)

For this thesis U-937 reporter cells were used, which were transduced beforehand with an NFκB-GFP Cignal lentivirus according to the manufacturer's instructions to generate NFκB reporter cells. Single cells were seeded and then expanded over the course of about 4 weeks.

For the ERK cell line, no reporter cells were used, since the transduced ERKKTR served as the signal output.

HEK-293 T

HEK293T cells are a commonly used human cell line in biological and medical research. These cells were derived from the HEK293 cell line, which was originally isolated from human embryonic kidney tissue. The “T” in HEK293T stands for “transformed,” indicating that these cells have been genetically modified to enhance their ability to grow in culture. They are derived from human embryonic kidney cells that were transformed with adenovirus type 5 DNA, resulting in their immortalization (*HEK293 Cells*, n.d.)

2.2 Establishment of cell lines

All receptor expressing cell-lines were generated using a third-generation lentiviral system. Based on the HIV-1 virus, lentivirus vectors were developed as an alternative to retroviruses for viral transduction, with the benefit that they can infect both dividing and non-dividing cells (Elegheert et al., 2018; Fuchsberger et al., 2023)

The third-generation lentivirus transduction system is an improvement over earlier generations of lentivirus-based delivery systems. It is designed to minimize the risk of unwanted immune responses and maximize the efficiency of gene delivery. This system includes three main components: a packaging plasmid (which is split into two plasmids: one encoding Rev and one encoding Gag and Pol), a transfer plasmid, and an envelope plasmid (*Addgene: Lentiviral Guide*, n.d.)

The packaging plasmid encodes all the viral genes required to produce infectious lentivirus particles, while the transfer plasmid contains the genetic material to be delivered into the target cells. The envelope plasmid encodes the viral envelope protein that allows the virus to enter and infect the target cells (*Addgene: Lentiviral Guide*, n.d.)

One of the key advantages of the third-generation lentivirus transduction system is its ability to accommodate large DNA payloads. This allows for the delivery of entire genes, rather than just small fragments, which is important for many gene therapy applications (Poletti & Mavilio, 2021).

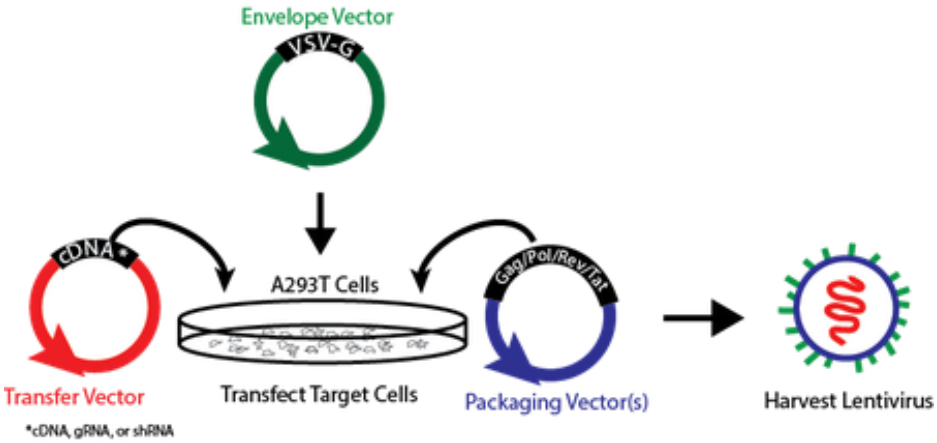

Figure 12: Production of lentivirions. It usually occurs in a mammalian cell line such as HEK293T or 293FT cells. The process initially involves co-transfecting host cells with recombinant lentiviral DNA constructs, which contain the viral genome and other viral proteins necessary for viral particle production. Once transfection has occurred, the viral particles are produced and harvested, typically by collecting the culture supernatant. (Figure adapted from: https://www.addgene.org/guides/lentivirus/)

For the transfection HEK293 cells were plated into a 10cm dish and cultured in conditions mentioned above until a confluency of around 80-90% was reached. To transfect the HEK cells, 2.5ug pMD.2G (Addgene #12259) as envelope plasmid, 6ug of psPax2 as packaging plasmid (Addgene #12260) and 10ug of PLV-GOI (pLentiPGK Blast DEST ERKKTRmRuby2 Addgene #90231; pLV[Exp]-Neo-EF1A>{Dectin1_SpyTag003} , hLangerin (constructed in Nina van Sorge lab)) were used. The genes of interest are under either the constitutive EF1a promoter (Dectin-1), or PGK promoter (ERKKTR). HEK293 cells were transfected using PEI (PEI is toxic for cells and the media was replaced 6 hours after transfection with fresh DMEM media) with vectors coding for the lentivirus and gene of interest (GOI). Lentivirions were generated for 48h and the supernatant frozen to kill any remaining HEK293T cells. Additionally, a quality control check was performed, where HEK293T cells were transduced with the produced virus. If the transduction efficiency was satisfactory, the crude extract (supernatant) was used to transduce the GOI into U937 cells *via* spin transduction at 900g and 33°C in the presence of 1µg/mL polybrene for 90 minutes. After 24h rest, the media was exchanged to complete RPMI and after another additional 48 hours the cells were selected with appropriate antibiotics (concentration was chosen based on the results of selectivity curves).

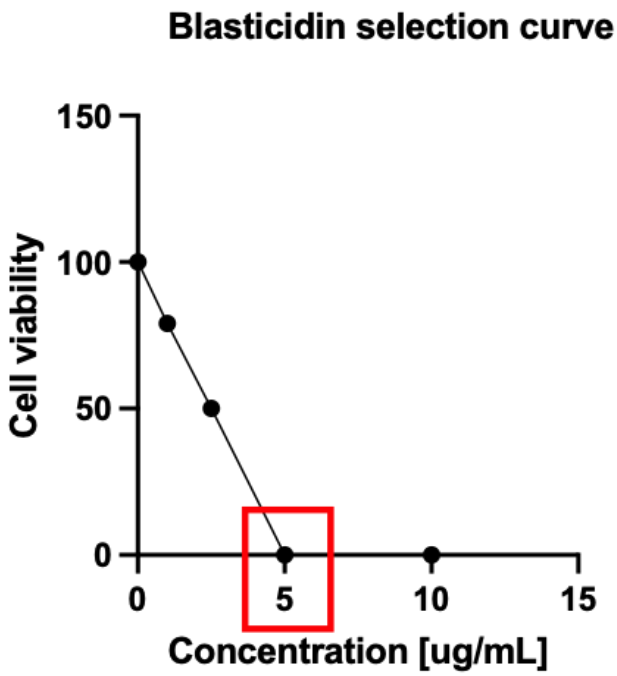

Figure 13: Selection curve for Blasticidin. U937 cells were treated with various concentrations of Blasticidin for 5 days and cell viability was measured afterwards. 5ug/mL was the lowest concentration that killed 100% of untransfected cells.

2.3 Antibody stain and detection

For surface stains, cells were first fixed in 4% PFA (Carl Roth) at 4°C for 20 minutes, then incubated in with the respective antibodies and isotype controls for 20 minutes at 4°C, and finally washed three times in FACS buffer before being measured via flow cytometry.

2.4 Preparation of SBG-lipid bilayer

For the preparation of the lipid bilayer, different molar ratios of DOPE and DOPC-cap-biotin were combined to get a titration of the biotinylated part (table 1). After carefully evaporating the Chloroform in the mixtures with Nitrogen gas, the lipids were placed in a vacuum desiccator for 30 minutes to ensure complete drying of the lipids. To the

tubes, 1mL ddH2O was added and then sonicated for 3 minutes (20s on, 10s off, 30% power). Meanwhile, the SiO2 beads (40uL) were cleaned in a piranha solution (3:1 ratio of sulfuric acid to hydrogen peroxide) for 30 minutes. After the cleaning, the activated SiO2 beads were washed three times and resuspended in 800uL ddH2O.

Table 1: Calculation of lipid ratio

		MW	c(stock), [mM]	c(stock), [mg/mL]	mol%	V total [uL]	V of lipids in Chl [uL]
0mol%	DOPE-cap-biotin	1053,00	9,50	10,00	0%	500	
	DOPC	786,00	63,61	50,00	100%		78,6
0.05 mol%	DOPE-cap-biotin	1053,00	9,50	10,00	0,05	500	0,13
	DOPC	786,00	63,61	50,00	99,95		39,28
0.1 mol%	DOPE-cap-biotin	1053,00	9,50	10,00	0,1	500	0,26
	DOPC	786,00	63,61	50,00	99,9		39,26
0.5 mol%	DOPE-cap-biotin	1053,00	9,50	10,00	0,5	500	1,32
	DOPC	786,00	63,61	50,00	99,5		39,10
1 mol%	DOPE-cap-biotin	1053,00	9,50	10,00	1	500	2,63
	DOPC	786,00	63,61	50,00	99		38,91
5 mol%	DOPE-cap-biotin	1053,00	9,50	10,00	5	500	13,16
	DOPC	786,00	63,61	50,00	95		37,34
7.5 mol%	DOPE-cap-biotin	1053,00	9,50	10,00	7,5	500	19,73

	DOPC	786,00	63,61	50,00	92,5		56.86
10 mol%	DOPE-cap-biotin	1053,00	9,50	10,00	10	500	52,65
	DOPC	786,00	63,61	50,00	90		70,74

2.5 Functionalization of Silica beads

Silica beads, each SUV, and MOPS buffer were combined, incubated for 15 minutes on a shaker, and washed ten times at 10.000g, with a final volume of 250uL. After the last washing step, 10ug/mL of labelled Streptavidin was added to each tube, incubated for 15 minutes, and washed ten times with a final volume of 250uL. The last step included adding different fractions of biotinylated SBGs (kind gift from Prof. Christensen) at a concentration of 25ug/mL and washing the mixture four times, having a final volume of 50uL (the last washing step was carried out in PBS). The final product was stable for two weeks.

2.6 Cell assays

Log phase reporter cells were being challenged in full media with the prepared functionalized beads for 16 hours or soluble beta glycans. After incubation, cells were measured via flow cytometry. About 10000 events were measured for every concentration step shown in the dose response curves or histograms.

2.7 Bacterial screen

To test the binding of bacterial glycans to cells and activation, cells were infected with an MOI of 20 for 16 hours. For this, several variants of two bacterial strains were used.

To measure the binding, strains were stained following the protocol of the Live Bacterial Gram Stain Kit Biotium #32000. After incubation, cells were fixed with 4% PFA at 4°, washed three times with PBS, and measured via Flow Cytometry. About 10000 cells were measured for every strain.

2.8 Bacterial titration

For the titration, four strains were chosen with the highest dose-response curve. The starting MOI was 50 and diluted 1:2 until an MOI of 1 was reached. The cells were incubated for 16 hours at 37°. After the incubation, cells were fixed with 4% PFA at 4° for 20 minutes, washed three times, and measured via Flow Cytometry.

2.9 Gating strategy

Cells were measured in PBS with a CytoFlex (Beckman Coulter, USA). Cells were concentrated not higher than 1e6 cells/mL. Recorded events were gated for single events and for their corresponding scatter pattern.

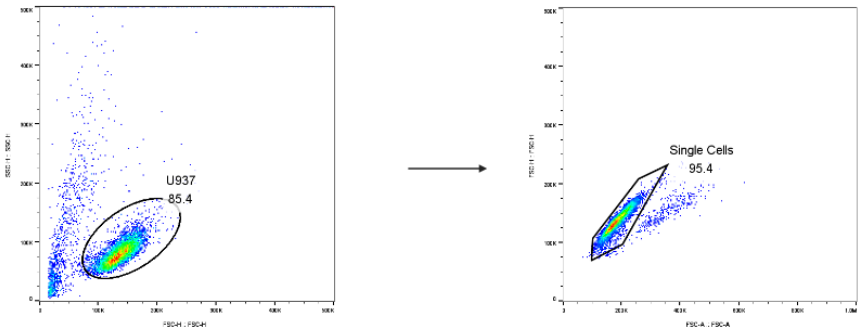

Figure 14: Gating strategy for U937 cells. Cells were first gated for live cells using FSC and SSC, excluding debris. Afterwards they were gated for single cells using FSC:A and FSC:H, excluding duplets

2.10 Immunostaining of Dectin1/NFkB

Beads (SLB tunable biotin, Sav (not labelled), SBG DP4-b) at tunable concentration were prepared one day before (SLB-Sav, but not SBG is added) and stored at 4°C overnight. Dectin 1 #5 cells were seeded in a 96 – well V shaped plate at 0.5x10E5 of cells per well. To this, 10ul of 5 times concentrated bead stock was added, centrifuged and incubated for 30 minutes at 37°. Afterwards, they were transferred to imaging chambers. Cells were fixed with 4% PFA in PBS for 20 minutes and washed three times. To permeabilize cells, 0.2% Tween was added and incubated for 10 minutes. The chambers were rinsed with PBS carefully (not to disturb cell attachment) and 3% (w/v) bovine serum albumin (BSA, ChemCruz) in PBS with 0.5 g/mL Fc Block (BD Biosciences) was added and incubated for 30 minutes to block non-specific binding. pSyk Alexa 488 was added to cells at 1:500 dilution in 1% BSA and incubated at room temperature for 1 hour. Cells were rinsed twice with 1% BSA, then incubated with anti-Dectin1- PE and anti-CD45-PE both at a dilution of 1:100 in 1% BSA for 1 hour at room temperature. Cells were washed 10 times with PBS before fluorescence and brightfield imaging with LSM980.

Average pixel intensity within the interface was quantified using an automated custom macro in ImageJ.

3. Material

3.1 Antibodies used

Table 2: List of antibodies

Antigen	Supplier	Product Number
anti-Dectin-1	BioLegend	355403
anti-CD207 h-Langerin PE	Miylteni	130-098-355
anti-CD45-PE	BioLegend	304007
pSyk Alexa488	BD Biosciences	560081
IgG2k	Biolegend	406001

IgG2a	Biolegend	407107

3.2 Lipids

Table 3: List of lipids

Lipid	Supplier	Product Number
DOPE-cap-biotin	Sigma Aldrich	384835-51-2
18:1 (Δ9-Cis) PC (DOPC)	Sigma Aldrich	4235-95-4

3.3 Vectors

Table 4: List of vectors

Vector	Supplier	Product Number
pMD2.G	Addgene	12259
psPax2	Addgene	12260
pLentiPGK Blast DEST ERKKTRmRuby2	Addgene	90231
pLV[Exp]-Neo-EF1A>{Dectin1_SpyTag003}	Vectorbuilder	

The vector for human Langerin was constructed and cloned by the group of Prof. Nina van Sorge.

3.4 Chemicals and buffer

Table 5: List of chemicals and buffers

Chemical	Vendor
BSA-b	Thermo Scientific
RPMI	Gibco
DMEM	Gibco
Glutamax	Gibco
PEI	ISTA
Tween	Sigma Aldrich

3.5 Bacterial strains

All strains were constructed from the group of Prof. Nina van Sorge at the University of Amsterdam as part of a collaborative project.

a. Staphylococcus aureus

- RN4220 wt
- RN4220 ΔtarM ΔtarS
- RN4220 ΔtarM ΔtarS + tarM
- RN4220 ΔtarM ΔtarS + tarP
- RN4220 ΔtarM ΔtarS + tarS
- USA wt
- USA ΔtarM ΔtarS
- USA ΔtarM ΔtarS + tarM
- USA ΔtarM ΔtarS + TarS

b. Streptococcus pyogenes

- Exp ΔgacI
- Exp ΔM
- Exp WT
- Stationary WT
- Stationary ΔgacI
- Stationary ΔM

3.6 Glucans

Soluble beta glucans (SBG) were kindly provided by Prof. Dr. Bjørn Christensen as part of a collaboratorive project between the University of Vienna and Norwegian University of Science and Technology.

Fractions of SBGs included:

- DP>19

- DP15-19
- DP 14
- DP 4

4. Results

4.1 Soluble SBGs can activate the NFkB pathway

As part of a collaborative project with Norwegian University of Science and Technology in Norway the lab of Bjorn Christensen extracted a set of soluble beta glucans (SBG) from *S. cerevisiae* cell walls. From the low molecular weight fraction (under 100 kDa), the group produced multiple fractions with different degrees of polymerization: DP>19, DP 15-19, DP 14, and DP 4 (Figure 15a). The degree of polymerization indicates the number of glucose residues present on the side chains of the SBGs (Fuchsberger, 2021). Upon recognition of Dectin-1, it was tested if the downstream signaling pathway of NFkB would be activated, by using a GFP reporter system, that could be detected by Flow Cytometry (Figure 15b). It can be seen in Figure 15c that the higher the input dose of each fraction, the higher the GFP signal, meaning that the downstream signaling pathway was activated in dose-response manner. To our surprise, in contrast to the previously published results, even the fraction DP4 that corresponds to b-glucan tetrasaccharides elicited an activation response, though it was lower than for the higher fractions. For the highest fraction, DP>19, the lowest concentration (1ug/mL) was sufficient to evoke a response, reaching a plateau relatively soon (Figure 15d). As expected, the two smaller fractions (DP15-19, DP14) showed a nice response titration, with the highest concentration reaching the highest response, similar to that of DP>19. This result clearly demonstrates, that soluble SBGs can activate the NFkB pathway in our reporter cells. Further work is required to investigate if the activation of NFkB pathway linked to the expression of corresponding cytokines (TNF-α and IL-1β) can be detected on primary cells (monocytes/macrophages) expressing Dectin-1 receptor.

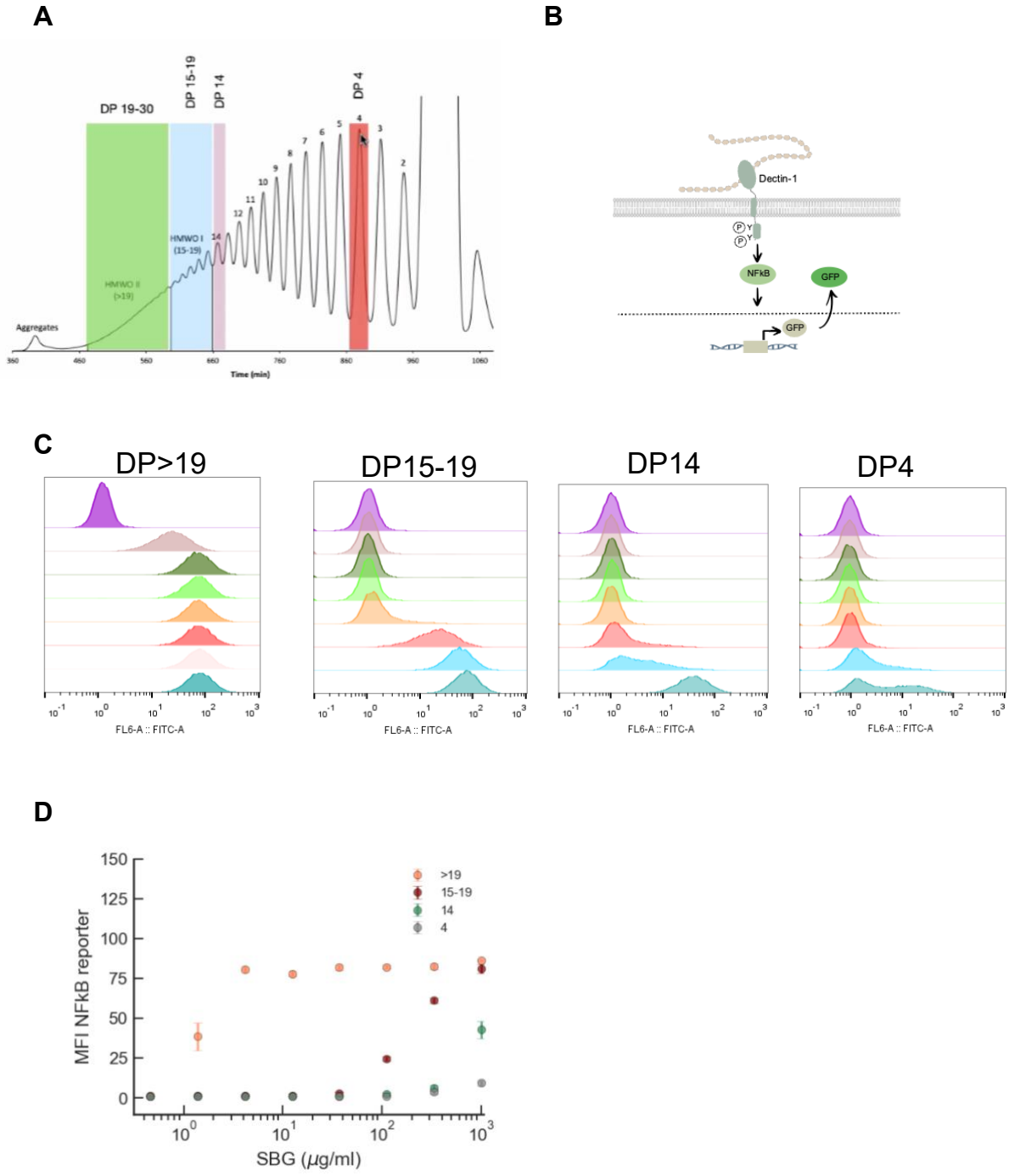

Figure 15: 15 (a) shows the fractination of soluble beta glycans (SBG) with DP4 being the smallest fraction obtained. (b) shows the generation of the NFkB reporter cell line and how it should work. Upon a stimulation of the Dectin-1 receptor, NFkB gets activated eading to a GFP signal, which can be detected via Flow Cytometry. The hypothesis is, the higher the input (glycan dose) the higher the output (stronger GFP signal). (c) depicts the raw data of U937-Dectin 1 cell line after activation via SBG for 16 hours. (d) shows the mean fluorescence intensity (MFI) measured via flow cytometry. The data represented here show the mean of n=3 experiments.

4.2 NFkB pathway can be activated by exposure to soluble β-glucan on biomimetic particle

Next, in order to mimic yeast particles, soluble beta-glucan were reconstituted on silica particles. To this end biotin-SBG were used (produced also in collaboration with the lab of Bjorn Christensen from NTNU in Norway) to functionalize silica particles with the following assembly: The first layer was a supported lipid membrane with the tunable density of DOPE-biotin, the second one consisted of Streptavidin and the final layer were biotinylated SBGs at different fractions.

By tuning the concentration of biotin-lipid, I was able to tune the density of SBG on the beads. Such tunable presentation allowed to quantify the minimal of the ligand to activate signaling via Dectin-1 receptor. Figure 16 shows a dose-dependent activation of Dectin-1 cells after exposure to particles functionalized with DP4. At the same time, the control experiment with the beads without SBG does not show any activation. Activation occurs in the cells with a very low density of DOPE-biotin lipid (thus low concentration of SBG-DP4) (0.01%). The activation can be divided into two ranges (0.00 to 0.1 and 1 to 10). The intensity of the GFP signal stays the same for the first four data points, whereas after that, a jump occurs, and the signal is significantly stronger, indicating a stronger activation. Again, it hit a plateau of intensity, meaning the saturation has already been reached. We found that even at low surface density of DP4 (260 ligands per μm^2) we can detect Dectin-1 activation.

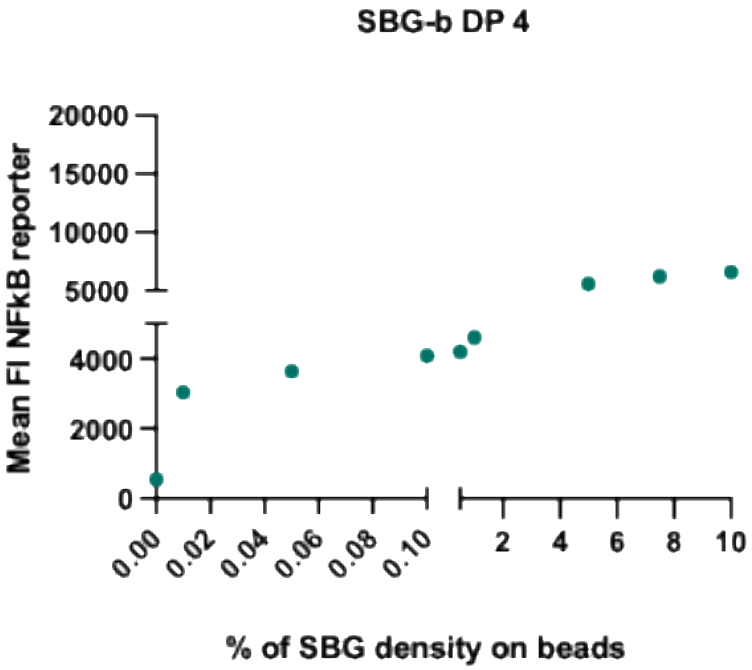

Figure 16: Activation of reporter cells upon exposure to biomimetic particles. The functionalized silica particles with the three layers were exposed to DP-4 for 16 hours and measured afterwards via Flow Cytometry. The data represented here show the mean of n=2 experiments.

4.3 BSA-b acts as a supportive layer

The activation at low density of DP4 could be linked to the fluidity of lipid bilayer which can support lateral clustering of tetrasaccharide ligand and therefore alter the results. For the non-fluid layer, we chose BSA-b, that can be easily deposited on negatively charged silica particles (Figure 17a). To control Dectin-1 specific activation by such mimetic particles U-937 WT cells were used that served as a negative control (Figure 17b right). The highest fraction of biotinylated SBG (SBG>19) has been chosen in this experiment. As expected, the functionalized beads with the lipid bilayer elicited a nice NFkB response. However, the non-fluid option with BSA-b showed similar results, already giving an NFkB signal at the lowest % of biotinylated BSA and thus lowest % of SBG density on the surface. Again, as in previous experiments (Figure 16), the activation via BSA-b as a supportive layer can be divided into two segments. The first activation plateau has already been reached at the lowest possible BSA-b concentration (0.01%) and stretches until 0.5% BSA-b density. Afterward, the MFI is

increased significantly, having a higher intensity than the particles with a supportive lipid bilayer and reaching another plateau there since the MFI for the highest possible BSA-b concentration (10%) is not significantly higher than the MFI for 5%. Contrarily, the particles with the lipid bilayer do not elicit a jump between the concentrations, but this system allowed better titration than the BSA-b system.

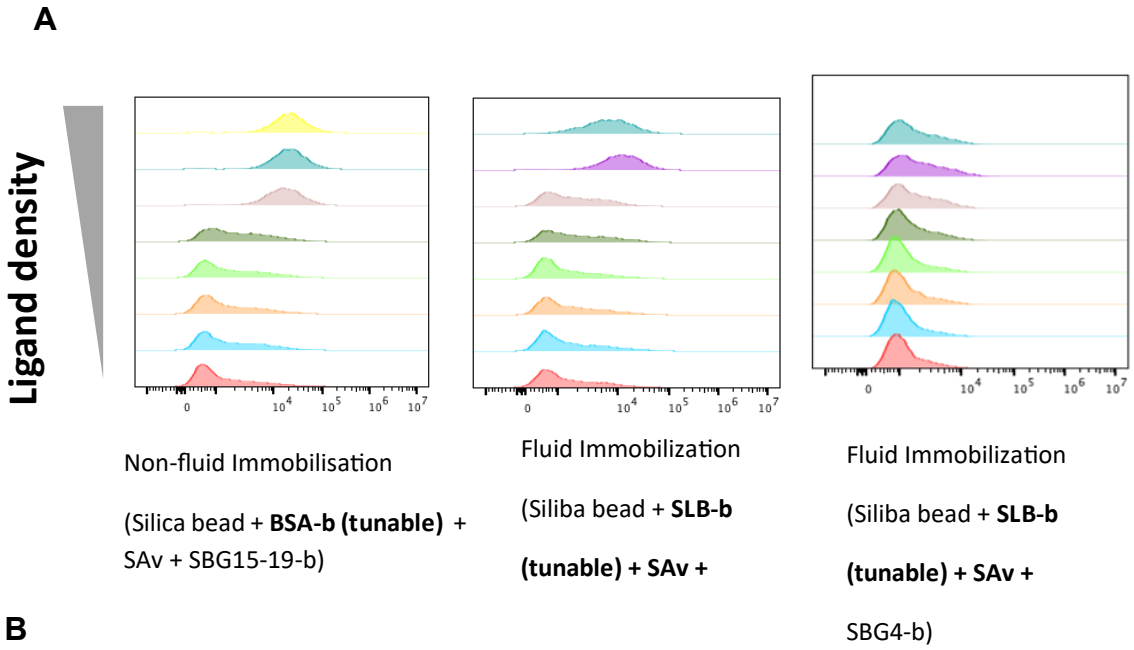

Figure 17: Comparison of activation between fluid (BSA-b) and non-fluid (lipids bilayer as a supportive layer. a) shows the representative data of fluid (middle and far right panel) and non-fluid (far left panel) immobilization of U-937 #5 Dectin-1 cells. Different fractions of biotinylated SBGs were used (SBG15-19-b, SBG4-b),(b) shows the NFkB activation of U-937 #5 Dectin-1 cell upon exposure to SBG DP>19 (left) the NFkB activation of wildtype U-937 #5 cells (right). The data show the mean of n=2 experiments.

4.4 Formation of immune synapse is not required for the activation of NFkB signaling

To visualize receptor distribution on U937 cells upon encounter with the mimetic particle the next step was to perform an immunostaining to see at surface density of b-glucan if an immune synapse is formed and how this correlates with the activation of NFkB signaling.

In Figure 18a, the cells were stained for two antibodies, CD45 and pSyk. Syk is a protein tyrosine kinase crucial for hematopoietic cells' intracellular signal transduction (Cheng & Chan, 1997; Chu et al., 1998) Immunoreceptor tyrosine-based activation motifs (ITAMs) are found in the cytoplasmic domains of immunological receptors, and Syk interacts with them. It links the immunoreceptors' downstream signaling activities, such as cellular proliferation, differentiation, and phagocytosis, to their activated state (Turner et al., 2000). Thus, pSyk staining should report with on the activation of NFkB pathways, since Syk phosphorylation is upstream in this pathway. CD45 is a membrane-associated glycoprotein, and it has been suggested that the cell surface phosphatase CD45 regulates the dephosphorylation of Src-family kinases (Donovan & Koretzky, 1993; Heath, 2001). In an immune synapse, Dectin-1 activation is possible because β-glucan particles induce clustering of the receptor at the site of contact. This clustering forms a phagocytic synapse, which physically excludes the tyrosine phosphatases CD45 and CD148. These phosphatases normally dephosphorylate signaling molecules necessary for initiating the immune response. When CD45 and CD148 are excluded, Dectin-1 can initiate signaling via Src and Syk kinases, leading to downstream immune responses like phagocytosis and ROS production. If CD45 is not excluded, it can dephosphorylate these kinases, preventing Dectin-1 signaling and thus inhibiting the immune response (Fuchsberger et al., 2023).

The analysis of the staining with Syk (green) and CD45 (violet) in Figure 18b supports the same conclusion. In both images, a cell is observed encountering the highest percentage of the biotinylated lipid bilayer, indicating the highest density of SBG-b. The cell stained with pSyk indicates a strong activation of downstream signaling events, such as NFkB. Surprisingly, even with a higher ligand spacing (lower ligand density), NFkB was still activated. This suggests that despite the absence of a formed

immune synapse, the immune signaling pathway was activated. Consequently, it is concluded that the formation of an immune synapse between Dectin-1 and the functionalized bead is not necessarily for the activation (see Figure 18).

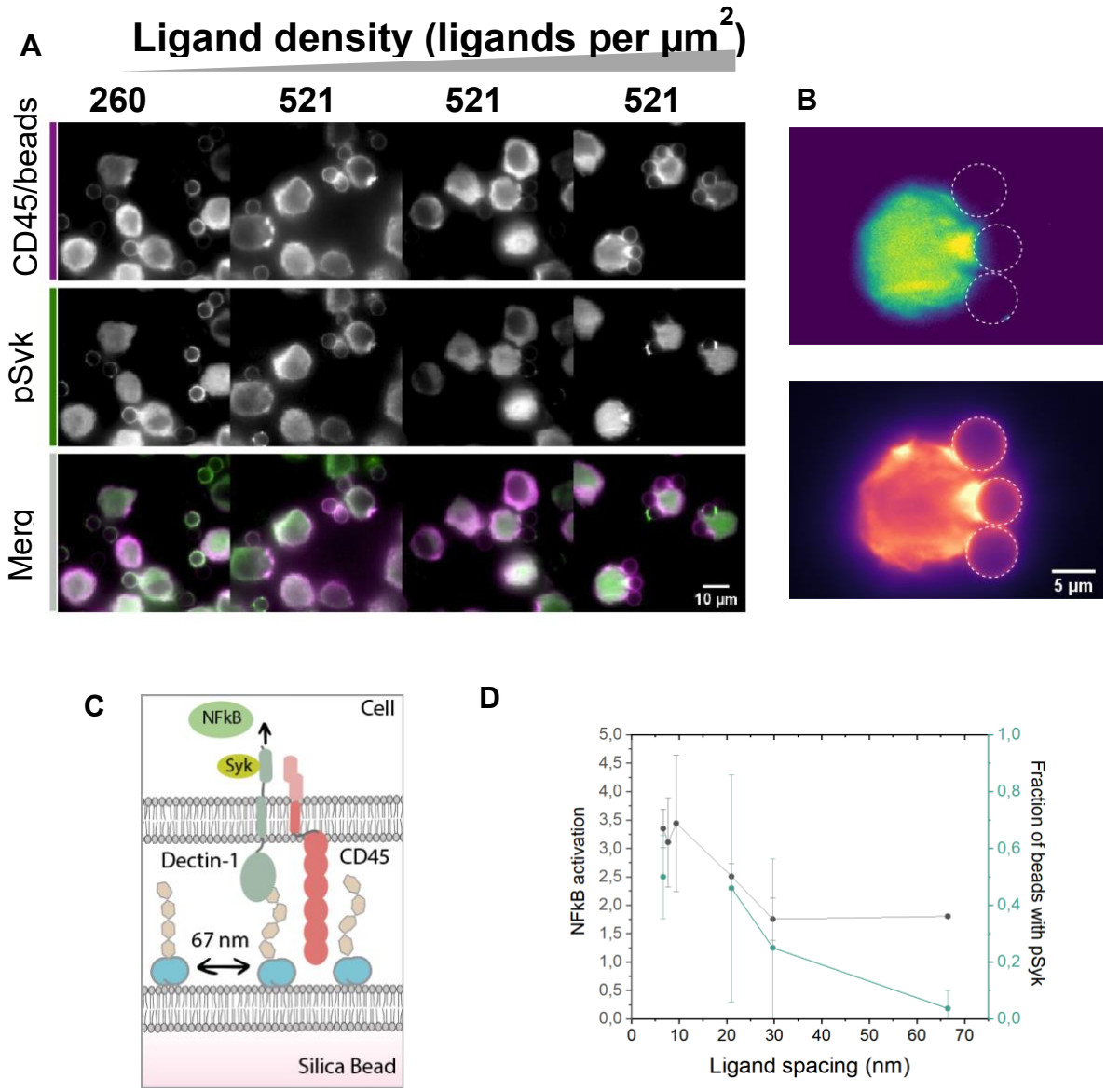

Figure 18: Immunostain of Dectin-1 upon exposure to biomimetic particles. (a) shows images of the cell upon encounter with the functionalized beads. The ligand density represents the tunable density of the biotinylated DOPE lipid. The upper row shows cells stained with CD45, the second row the channel for cells stained with pSyk and the last row merges both channels. (b) shows one cell at closer look upon activation with DP>19. The upper picture shows the channel for CD45, the lower picture for the pSyk channel. (c) depicts a representative scheme of the ligand spacing and activation of Dectin-1 after encountering the functionalized silica beads. (d) shows the data for the mean NFkB activation with n=1 experiments. All images were taken with the Zeiss Elyra and evaluated via ImageJ (Figure courtesy of: Natalia Baranove, PhD)

4.5 Soluble beta-glucans can activate the MAPK pathway

Transitioning from NFkB reporter cells, real-time activation experiments were conducted using U937 ERK cell line, which were previously established by me. U-937 cells containing the ERK-KTR sensor and additional Dectin-1 Spytag were exposed to soluble beta-glucan fragments, since they activated NFkB reporter cells (Figure 15c). An ERK-KTR (Extracellular signal-Regulated Kinase Kinase Translocation Reporter) sensor is a genetically encoded biosensor designed to monitor the activity of the ERK signaling pathway in living cells. This sensor typically includes a fluorescent protein, such as GFP, fused to a fragment of a protein that can be phosphorylated by ERK. This fragment also contains a nuclear export signal (NES), which regulates the sensor's localization within the cell. When ERK is inactive, the sensor stays in the nucleus because the NES is masked. Upon ERK activation, the sensor gets phosphorylated, which unmasks the NES and causes the sensor to move from the nucleus to the cytoplasm. This change in localization can be observed using fluorescence microscopy (de la Cova et al., 2017). For this experiment, the highest possible fragment (DP>19) was selected to ensure activation of the MAPK pathway. Time-lapse imaging was performed using confocal LSM980 for 30 minutes, with images captured every five minutes. The nucleus of the cell was stained with Hoechst dye (yellow) to differentiate between the nucleus and cytoplasm and facilitate analysis of activation.

Comparing timepoint 0 (the initial image in Figure 19a) with timepoint 6, taken 25 minutes after exposure to soluble beta-glucan (the final image in Figure 19a), it is evident that the ERK signal (depicted in blue) has diminished within the nucleus and dispersed throughout the cytoplasm.

The depletion of the ERK signal is further confirmed after zooming in into one cell at timepoint 6 (Figure 19b). The ERK signal in the far right picture is depleted in the nucleus, confirmed with the second picture depicting where the nucleus is located, and moved into the cytoplasm, forming a nice ring around the nucleus. This result, combined with Figure 19a, confirms the real-time activation of the cells. Therefore,

soluble beta glycans are also capable of activating the MAPK pathway. However, to compare and conclude further studies are necessary.

Furthermore, as seen in the microscopic pictures it is revealed that the signal observed was weaker than anticipated, albeit still visible and distinguishable. To draw accurate conclusions, an improvement of the signal would be necessary. Figure 19a shows that the cells constantly move, making it hard to film for more than 30 minutes. One significant improvement would be to make the cells adhere beforehand by coating the plates with Laminin or Poly-L-Lysine. However, one needs to be careful to evaluate if the cells would have an unspecific activation by this coating.

Additionally, it was noted that the overall density of Dectin-1 on the ERK cell line was one-third lower than that of the NFkB cell line (Figure 20). The reduced receptor density may account for the relatively weak activation of the MAPK pathway and the overall diminished signal. Despite the weaker activation compared to the NFkB reporter cell line, it remained noticeable, leading to the conclusion that soluble beta-glucans can activate the MAPK pathway via Dectin-1.

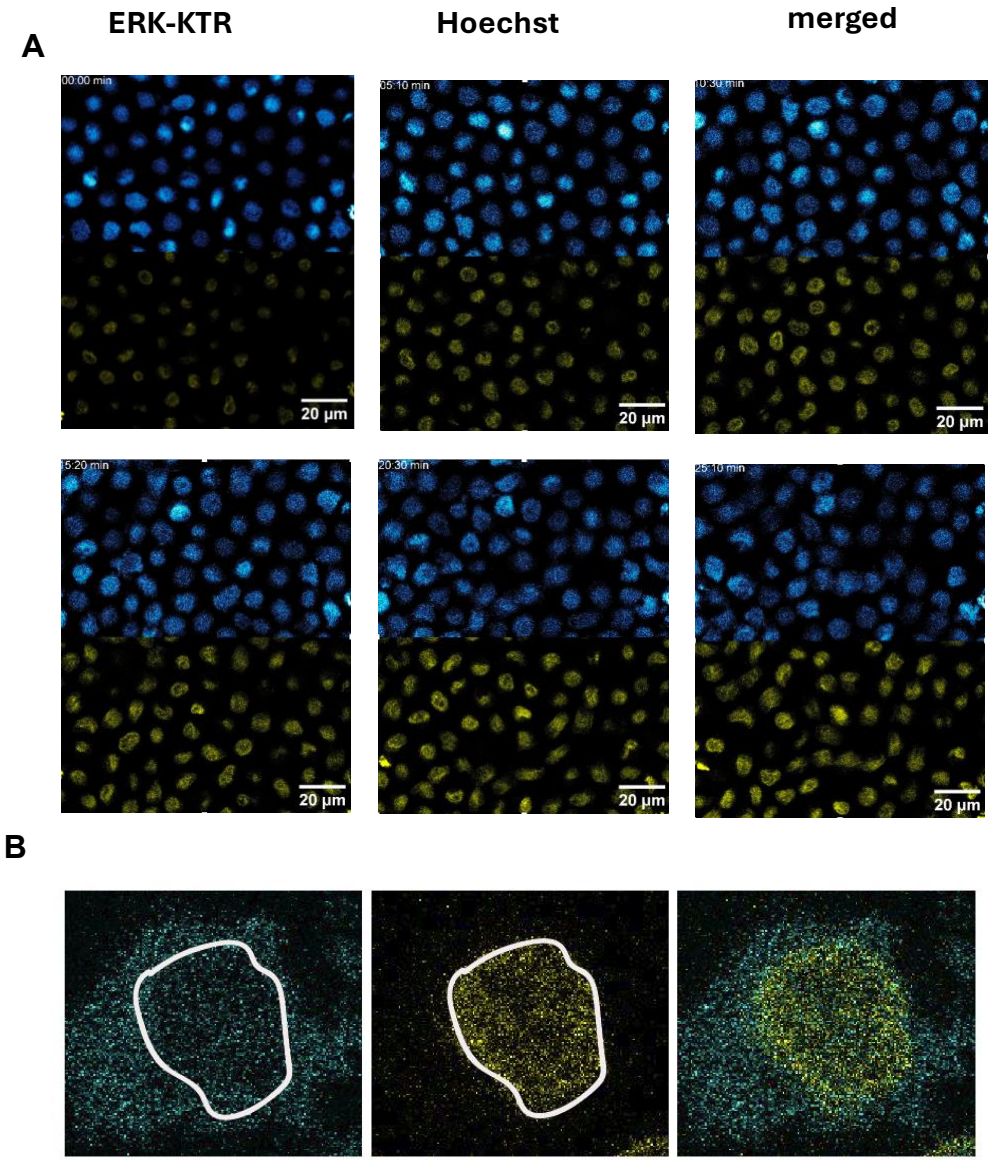

Figure 19: Real time activation of U-937 ERKKTR Dectin-1 cells upon exposure of SBG DP>19. (a) The shots are taken every 5 minutes (b) shows one cell after activation, where the ERK signal (blue) in the nucleus (yellow) is depleted after activation. The right picture combines both signals coming from the nucleus and cytoplasm.

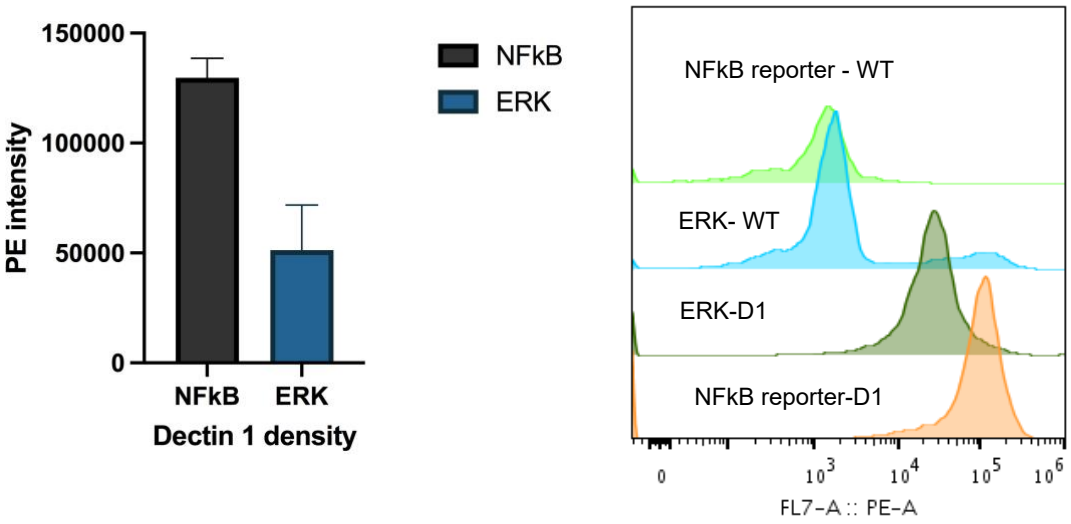

Figure 20: Comparison of Dectin-1 density between U-937 NFkB reporter cells and U-937 ERKKTR cells. The right picture schows the raw data obtained with the Flow Cytometer. Here both wildtype and Dectin-1 expressing cells were measured. On the left, the density of Dectin-1 is compared between the NFkB and ERK reporter cells. The data show the mean of n=3 experiments.

4.6 Bacterial glycans activate the immune signaling via Dectin-1/Langerin

The second part of the result section deals with recognizing bacterial glycans via CTLs.

To determine whether human Dectin-1 and/or human Langerin (alone or as a double positive) can bind and recognize glycans of the bacterial cell wall, several engineered variants of two gram-positive bacteria, namely Staphylococcus aureus and Streptococcus pyogenes, were used and exposed to the reporter cells for 16 hours and measured afterward via flow cytometry. The data was converted into two heatmaps (Figure 21 and 22), with binding (red heatmap) and activation (green heatmap) via the respective CTL being shown.

At first glance, two variants especially trigger an NFkB response, namely the wildtype in stationary and exponential phase, which is not surprising since the other variants are either missing the M glycoprotein or the gacI. M protein is the major virulence factor

of S. pyogenes and can bind to several receptors. Without the M protein, the bacteria are more likely to be unnoticed by the receptor. Moreover, comparing Dectin-1 with the double positive cell line Dectin-1/hLang, human Langerin as an additional receptor amplifies the NFkB signal upon exposure to the bacteria. More interesting is that human Langerin alone already elicits a response to the strains. This is surprising, as according to the literature, human Langerin does not contain an ITAM motif (del Fresno et al., 2018), which is necessary to activate downstream signaling pathways. In the case of the stationary Streptococcus pyogenes strain, the cells expressing the human Langerin receptor have an even stronger response than those with only the Dectin-1 receptor. In all cases, however, human Langerin has the most decisive response to the strains. This correlates with the results when measuring the binding to the different strains. Especially the stationary wild type seems to be triggered by human Langerin and is even further enhanced as a double-positive receptor cell line. The weakest response comes from exponentially and stationary variants where the virulence factor M needs to be added. One reason, as mentioned above, is that the M protein is the major virulence factor of the bacteria. With that being missing, the receptor is less likely to bind to the bacterial cell wall. Comparing the results of the CTR with the negative control, in this case, wildtype U-937 cells, however, it can be seen that the wildtype cells as well do bind to the bacterial strains (Figure 21b) but do not elicit an activating response for NFkB. This enhances the hypothesis that Dectin-1 can be activated via bacterial glycans. Moreover, human Langerin, up to date not known to have an activating function, enhances this, and can function on its own as a receptor with activating functions.

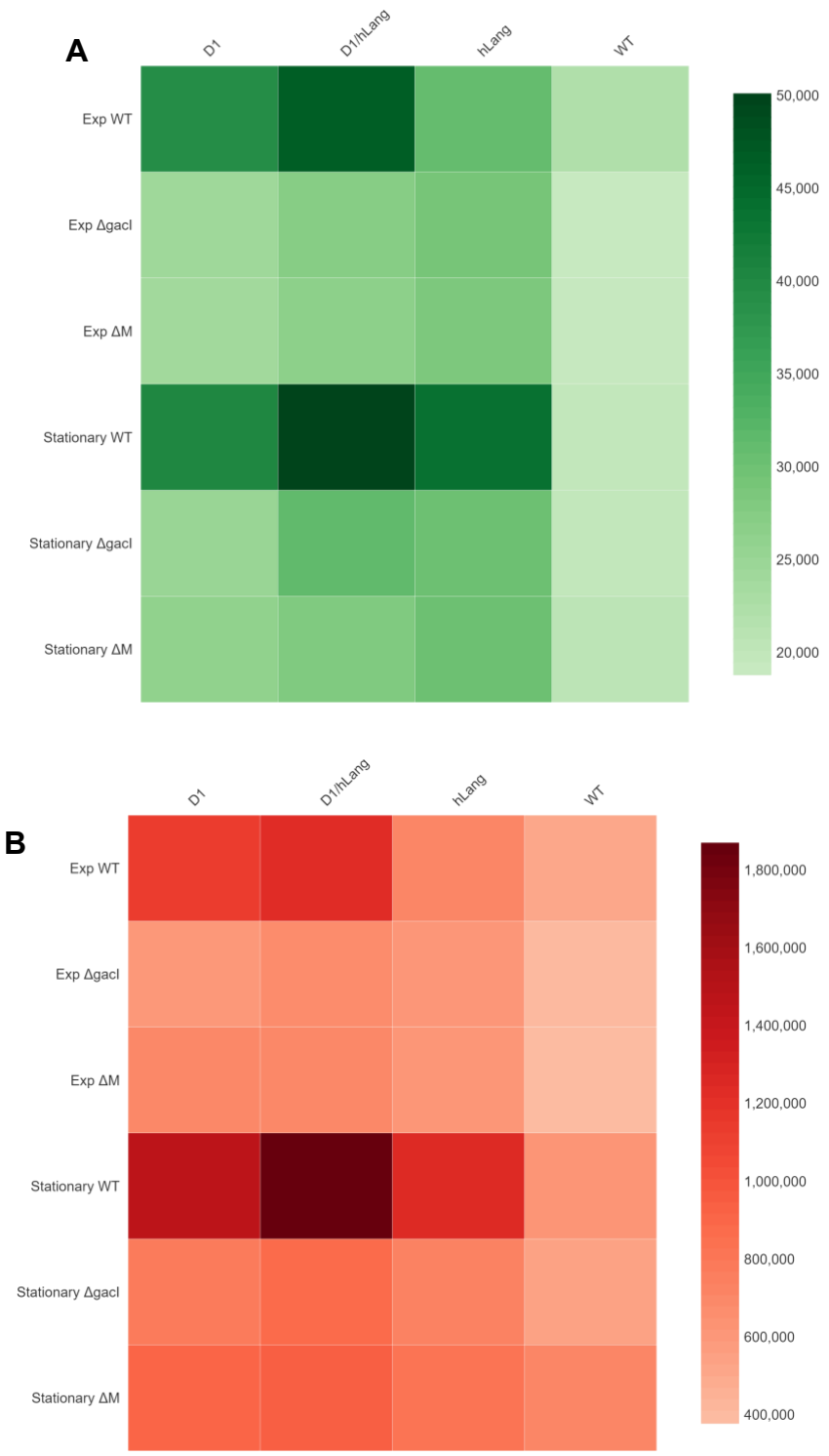

Figure 21: Heatmap representing the NFkB activation (a) and binding (b) via C-type lectin receptors of variants of the gram-positive bacteria Streptococcus pyogenes. The data show the mean of n=3 experiments. The cells were exposed to stained bacterial strains for 16 hours, washed and fixed in PFA, and measured via Flow Cytometry. Afterwards the raw data was analyzed via Python.

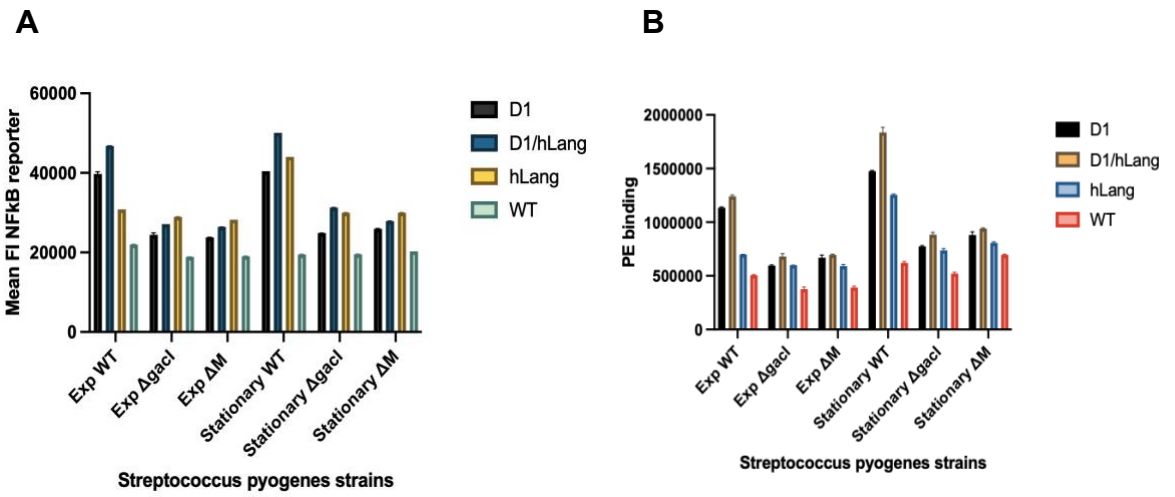

Figure 22: Activation of different CTR via glycans of Streptococcus pyogenes. (a) shows the MFI of the NFkB reporter, (b) the MFI of the binding strength to the glycans. Different variants were tested, including bacteria in the stationary and exponentially phase either missing the M or gacI protein. The data shown represents the mean of n=3 experiments.

For the variants of the Staphylococcus strain, especially three variants stand out, namely RN4200 wildtype, RN4220 ΔtarMΔtarS+tarP, and RN4220 ΔtarMΔtarS + tarS.

In all four cases, Dectin-1 has the same or slightly less NFkB activity (Figures 23 and 24) than the double positive cell line Dectin-1/human Langerin. Although the mutant RN4220 strain with deletions of both tarM and tarS elicits the highest activation with cells having Langerin as their receptor, this does not correspond to the double-positive cells. Although the density of human Langerin is lower, one could have expected a higher activation here. However, in all these cases, the binding is significantly increased in cells having Langerin and Dectin-1 as receptors (Figures 23and 24). As in previous studies, binding of Langerin to wildtype RN4200 with double mutation was slightly reduced, especially noticeable for the cell line expressing both Dectin-1 and Langerin. Binding could be restored by complementation with tarM and tarS, but not with tarP. In contrast, completion with tarP resulted in a more reduced binding efficiency compared to the double mutant strain.

Comparing both binding and activation of all cell types, it can be said that although the binding is increased when having two receptors, the activation is not necessarily with those variants of Staphylococcus aureus. Like for Streptococcus pyogenes, cells with only human Langerin can also trigger the highest NFkB activation. Wildtype cells show

a significantly higher binding capacity to Staphylococcus strains than to Streptococcus strains, specifically RN4220 wildtype and ΔtarM ΔtarS. However, as expected, they do not show any activating function of NFkB. The high binding efficiency of wildtype cells can be explained by the fact that for the staining, WGA (wheat germ agglutinin) was used, a lectin that can be used for cell membrane labeling, as it can bind to almost every CTL.

For some variants, the background noise is relatively strong, however looking at the variants mentioned above, namely RN4220 ΔtarMΔtarS+tarM and RN4220 ΔtarMΔtarS + tarS, the WT cells barely bind to the bacterial cell walls, as well as Dectin-1 with a weak binding capacity. Only the receptor Langerin can bind to those variants efficiently, also reflected in the NFkB response. Interestingly, although the variant where tarP was added (RN4220) does not seem to be bound by the receptors, it still can elicit an NFkB activation. The opposite can be observed for the variant where tarM was additionally added (RN4220). Although the receptors bound to these bacteria, especially the double positive cells, the NFkB activation for this cell line does not reflect it. Moreover, overall, the binding for the USA strains was weaker than for RN4220, which partially is reflected in the NFkB response as well.

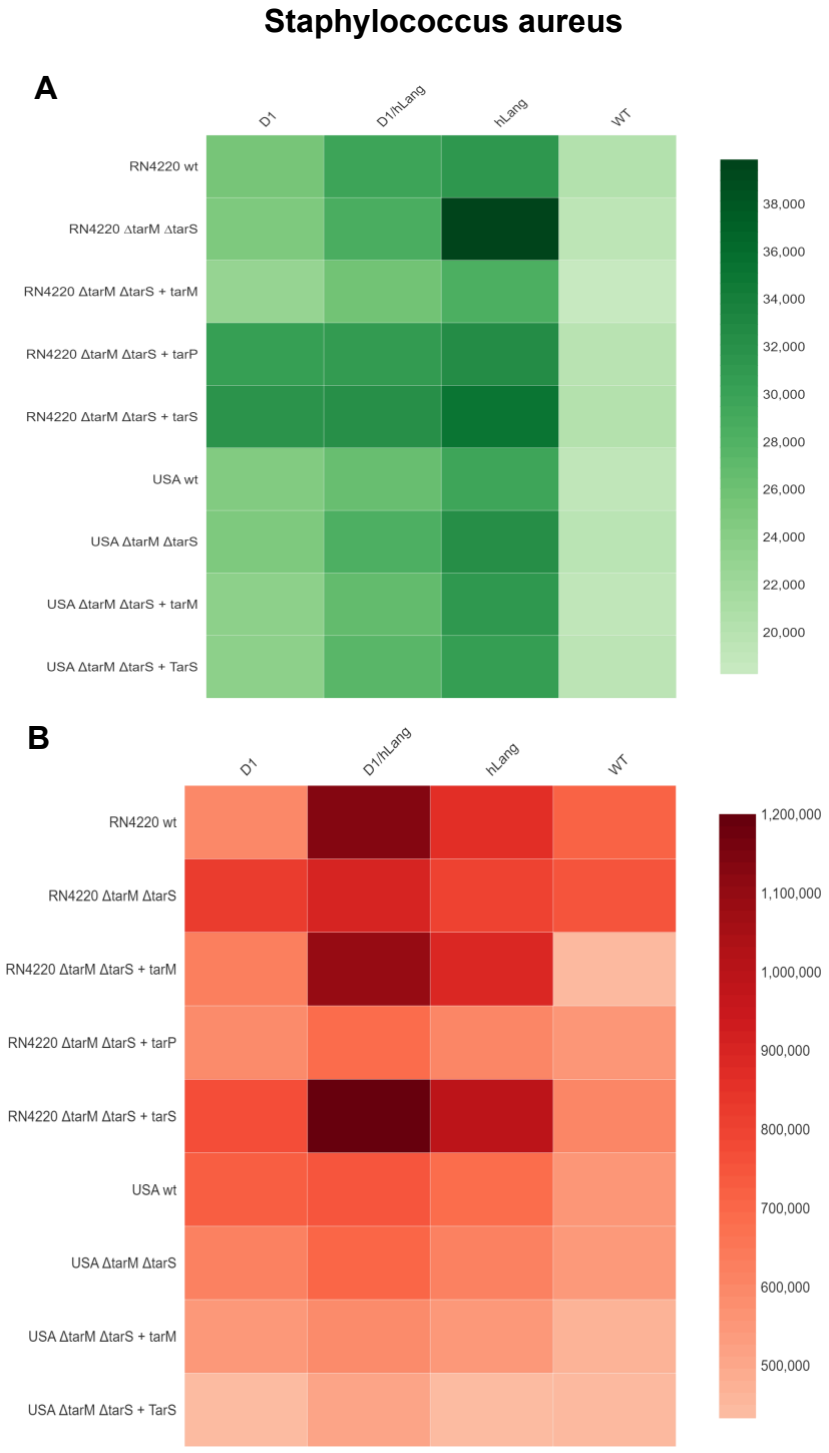

Figure 23: Heatmap representing the NFkB activation (a) and binding (b) via different C-type lectin receptors of variants of the gram-positive bacteria Staphylococcus aureus. The data show the mean of n=3 experiments. The cells were exposed to stained bacterial strains for 16 hours, washed and fixed in PFA, and measured via Flow Cytometry. Afterwards the raw data was analyzed via Python.

Figure 23: Activation of different CTR via glycans of Staphylococcus aureus. (a) shows the MFI

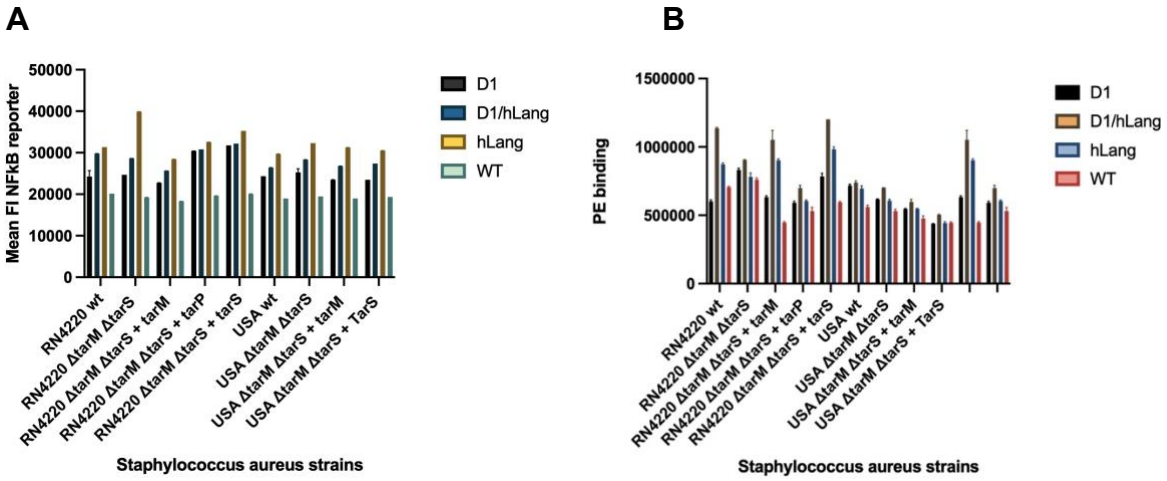

Figure 24: Activation of different CTR via glycans of Staphylococcus aureus. (a) shows the MFI of the NFkB reporter, (b) the MFI of the binding strength to the glycans. Different variants were tested, including bacteria missing the glycotransferases tarM, tarS and tarP. The data shown represents the mean of n=3 experiments.

4.7 Bacterial titration

Lastly, a titration was carried out of two different variants of Staphylococcus aureus (RN4220 ΔMS+S, ΔMS+P) as well as two different variants of Streptococcus pyogenes (WT exp and WT stat) to inspect when the NFkB activation starts taking place and if a low MOI (multiplicity of infection) is already enough for that. The starting MOI was 50; with 1:2, dilution went down to MOI 1. Only #5 Dectin-1 cells were tested for this titration.

Figure 25a shows the raw data of activation and binding upon exposure of gram-positive bacteria. One can notice that, especially for the highest MOI of 50, the raw signal looks weak and almost non-existent, mainly when focusing on activation. This indicates that with MOI50, the cells have already reached a plateau and are oversaturated with bacterial glycans. The cells cannot function properly and are more prone to cell death, which the low viability count from the Cytoflex also indicated. This can also be observed in Figure 26 and 27, where the mean of n=3 experiments is represented. Significantly for both S. pyogenes strains, a visible cut can be seen

between the lower MOIs and MOI 25 and 50. MOI50 does not elicit a significantly higher NFkB response via Dectin-1, leading to the conclusion that a higher MOI does not necessarily mean an increased response. No differences can be observed between the stationary and exponential phases as well. For the S. aureus strains, it is noticeable that with an increasing MOI, the MFI increases as well, reaching its high only at the highest possible MOI without being saturated. MOI25 (second highest) elicits an activation half that strong, which is to be expected. Something noticeable is, that deleting the WTA tarS does not have an impact on the recognition of bacterial cell wall via lectin receptors, since both variants have comparable results. Comparing the NFkB activation to the binding efficiency of all strains, even though the respective MOI did not elicit a response, the receptor was still able to bind to the strain. However, saturation was also reached earlier for S. pyogenes strains.

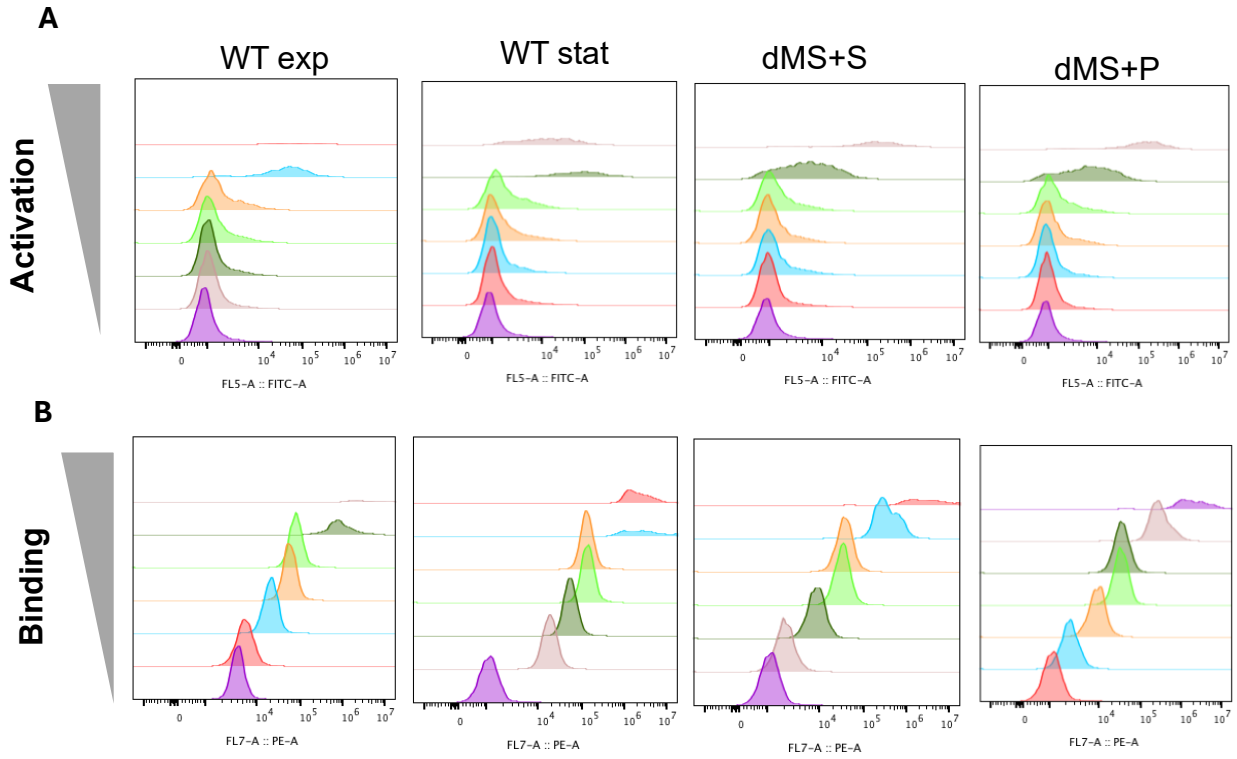

Figure 25: Bacterial titration of chosen variants. (a) shows the raw representative data of the NFkB activation. U-937 #5 Dectin-1 cells were exposed to four variants at different MOI (50 and then 1:2 dilution until MOI of 1 was reached) for 16 hours, washed, fixed with PFA and analyzed via Flow Cytometry. (b) shows the raw representative data of the binding of bacterial glycans via Dectin-1.

Streptococcus pyogenes

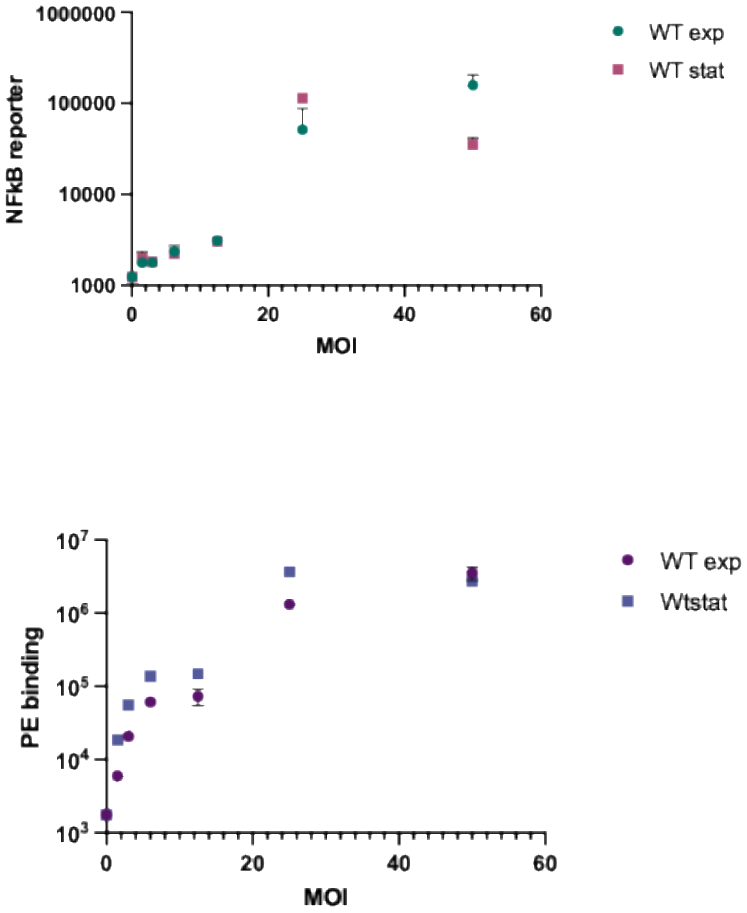

Figure 26: Bacterial titration of chosen variants. U-937 #5 Dectin-1 cells were exposed to four variants at different MOI (50 and then 1:2 dilution until MOI of 1 was reached) for 16 hours, washed, fixed with PFA and analyzed via flow cytometry. This figure represents the mean of n=3 experiments for all strains.

Staphylococcus aureus

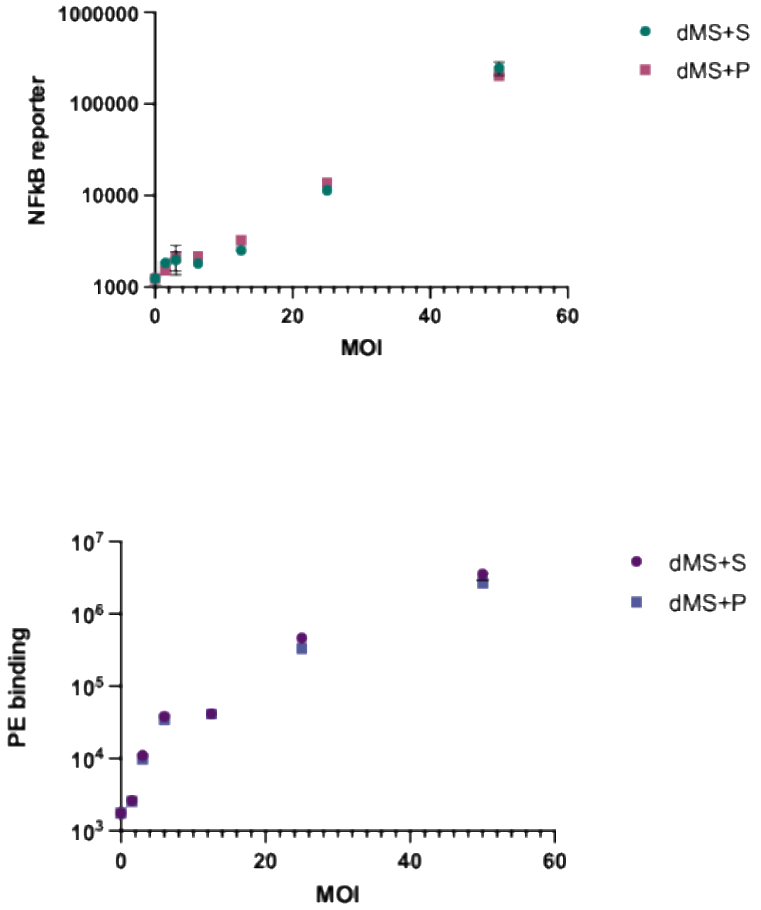

Figure 27: Bacterial titration of chosen variants. U-937 #5 Dectin-1 cells were exposed to four variants at different MOI (50 and then 1:2 dilution until MOI of 1 was reached) for 16 hours, washed, fixed with PFA and analyzed via flow cytometry. This figure represents the mean of n=3 experiments for all strains.

5. Discussion

The main goal of this work was to investigate the signaling potential of both C-type lectin receptors, Dectin-1 and Langerin. Therefore, reporter cell constructs were created, and several in vitro experiments were conducted to simplify massive signaling cascades and all interactions to inform us about the final hit-gene activation.

Overall, the results suggest that low concentrations of soluble beta fragments can already activate the NFkB and the MAPK pathway (Fig. 12). It demonstrated before that only yeast particles can activate Dectin1 signalling leading to the production of interleukins (Goodridge et al., 2011). Such activation requires the formation of immune synapse, with the exclusion of CD45 phosphatase and clustering of Decin1 receptor. However, my goal was to understand how C-type lectins decode glycans, and how spatial presentation of glycans affects their downstream signaling. During that I could prove that with well-defined glycans and a signaling sensor, that short soluble fragments (tetrameric beta-glucans) can activate NF-kB and MAPK/ERK- singaling pathways as well. To further confirm this result, BSA-b was chosen as a non-fluid layer to exclude the possibility that the fluiditiy of the lipid bilayer altered the results. With BSA-b the activation was reached even faster, with the first saturation possibly being the activation and the second one potentially being activationsand phagocytosis.

To explain this strong discrepancy, I suggest that the recognition of beta-glucans can induce some degree of Dectin1 oligomerization that is already sufficient to activate immune cells without a need to form a stable immune synapse (Figure 28). To prove this, more work has to be done in the future. Additionally, I demonstrated that one does not need yeast particles for Dectin-1 to oligomerize but can achieve this with soluble beta fragments at different fractions (with the fraction DP>19 giving the best yield) (Figure 15-19).

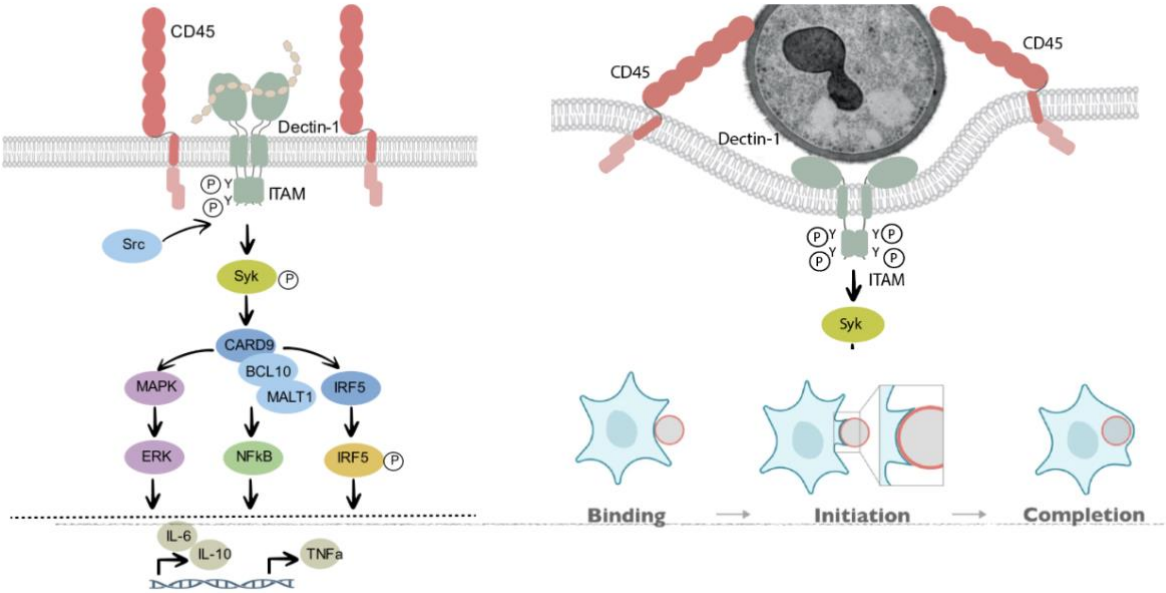

Figure 28: Schematic representation of the Dectin-1 signaling and the formation of the phagocytic synapse . When bound to fungal particles, it activates a signaling pathway that leads to the production of cytokines and other immune molecules. The important components of this pathway includes the protein spleen tyrosine kinase (Syk), the adaptor protein CARD9, and the transcription factors NF-κB and AP-1. (Figure courtesy of: Natalia Baranova, PhD)

Moving on from soluble fragments to biotinylated fragments, I was able to functionalize silica particles to tune the density of biotinylated SBGs. Using this tunable reconstitution, my goal was to quantify the minimal surface density and spatial arrangement of soluble-beta glucans required to induce activation of the downstream signaling via the Dectin1 receptor. For this, fluorescence microscopy was used. However overall, the dynamics of this interaction between Dectin-1 and the functionalized beads are relatively weak. Although activation of the NFkB and MAPK pathways could be observed (Fig. 15 to 19), especially for the lower fractions, the activation is weak.

Moreover, as seen in the microscopic pictures, the signal is weaker than expected, although visible and distinguishable. Here, an improvement of the signal is necessary for it to draw proper conclusions. Switching from confocal microscopy with PSD detectors to Eppi has already improved the imaging.

A very limiting factor of these experiments was that the cell line used was in suspension. Figure 19a shows that the cells constantly move, making it hard to film for more than 30 minutes. A significant improvement would be to make them adherent beforehand by adding Fibronectin-b to the surface, again to make live-cell imaging easier. Although the cells got adherent upon recognizing soluble beta-glucans, it was hard to track down a single cell initially. If the cells were adherent, TIRF imaging could be used to track down single cells. Moreover, although the transduction of Dectin-1 Spytag was successful, the Dectin-1 density was a third lower in those cells than in the reporter cells (Figure 20). This ultimately led to a decreased signal and more challenging tracking of cells in live cell imaging. One approach could be using a cell line with Dectin-1 already expressed on its cell surface. This would decrease the workload and ensure that the density stays consistent. This lower receptor density can explain the relatively weak activation of the MAPK pathway and the weak signal overall. Although the activation was weaker than for the NFkB reporter cell line, it was still noticeable, concluding that soluble beta-glucans can activate the MAPK pathway via Dectin-1. Another point, although the results suggest that soluble beta glycans are also capable of activating the MAPK pathway, to compare and conclude further studies, it is necessary to try to activate the MAPK pathway with all soluble fragments, not only the largest (as it was expected to have an activating function). It would be interesting to see if the smallest soluble fragment (DP 4) affects the MAPK pathway similarly to the NFkB activation.

The most exciting aspect regardless, of this work is the result indicating direct signaling from Langerin receptor. Human Langerin has not yet been described in the literature as a receptor with an activating function. With ITAM motifs missing in the Langerin case, it was assumed that also NFkB activation would be lacking, as well as other signaling pathways. However, this thesis's results suggest that this receptor is a CLR with an activating function (Fig 20-22). Further studies are needed for Langerin as this receptor could also play a fundamental role in targeted drug delivery via the skin.

6. Conclusion & Outlook

The main goal of this work was to understand how glycan presentation affect receptor signaling, if and how the combination of receptors would affect the signaling outcome and to develop novel microscopy assays to monitor such interactions, focusing on two receptors, namely Dectin-1 and Langerin.

Overall, the results suggest that small concentrations of soluble beta fragments can activate the NFkB and the MAPK pathway. However, a formation of the immune synapse is not necessary for cytokine production, which was unknown of such up to date. Moreover, it is proven that one does not need yeast particles for Dectin-1 to oligomerize but can achieve this with soluble beta fragments at different fractions (with the fraction DP>19 giving the best yield). Combining both receptors led to an increased NFkB-signaling, upon activation with bacterial glycans, even with mutated ones.

The combination of the reconstitution and cell microscopy approaches was the start to provide a new dimension to explore intracellular communication through the glycans. That is so far a unique approach for the field of glycobiology/glycoimmunology. To further develop this assay, information theory can be integrated. By integrating this, one can investigate how specific spatial presentations such as nanoclusters affect intracellular communication involving glycans and the bulky properties of bacterial glycans like WTA, LTA, LPS, and capsule polysaccharides. This approach can shed light on the mechanisms behind the activation of innate immune responses through various lectin receptors (in my example, Dectin-1 or Langerin). This will further allow one to quantify soluble-beta glucans' minimal surface density and spatial arrangement. Moreover, this would allow a completely novel assay with direct applications that will inspire the development of novel glycomimetics for drug delivery via the Dectin-1 receptor. Because Dectin1 is a signaling receptor, we would be able to quantify conditions and density of glycan ligand that lead to controlled production of interleukins by monocytes. In this way, we can engineer immune cell response to achieve a particular level of activation in drug delivery or vaccine application.

Overall, this study highlights the crucial roles of Dectin-1 and human Langerin in immunology. Historically, Dectin-1 has received limited attention; however, an increasing number of researchers are now recognizing its significant potential for translational science. The discovery that Dectin-1 can be activated by soluble β-glucans opens up new research possibilities, especially for using these β-glucans as adjuvants in immunotherapy to enhance therapeutic efficacy. Generally speaking, C-type lectin receptors (CLRs) have primarily been investigated for their roles in antifungal immunity. Nevertheless, the results presented underscore the significance of CLRs in bacterial immunity as well, highlighting the need for further investigation into the activation mechanisms of human Langerin. These findings could be an important first step in expanding the use of these receptors for new treatments, especially for developing vaccines.

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