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Immunology Unit &
Research Center for Emerging Infections and Zoonoses

Role of C-type lectin receptors in bacterial recognition

THESIS
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“Imagination is more important than knowledge“

- Albert Einstein-
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* T. Matsumura and S. Mayer-Lambertz contributed equally as joined second authors to this work.

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<tr>
<td>ABTS</td>
<td>2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt</td>
</tr>
<tr>
<td>AIM 2</td>
<td>absent in melanoma 2</td>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>AP 1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>BMDC</td>
<td>bone marrow-derived dendritic cell</td>
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<td>BMM</td>
<td>bone marrow-derived macrophage</td>
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<tr>
<td>CARD9</td>
<td>caspase recruitment domain family member 9</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>CHO</td>
<td>chinese hamster ovary</td>
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<td>Clec</td>
<td>C-type lectin</td>
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<td>CLR</td>
<td>C-type lectin receptor</td>
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<tr>
<td>CpG DNA</td>
<td>CpG oligodeoxynucleotides</td>
</tr>
<tr>
<td>CRD</td>
<td>carbohydrate-recognition domain</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
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<tr>
<td>CTLD</td>
<td>C-type lectin-like domain</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DCAR</td>
<td>dendritic cell immunoactivating receptor</td>
</tr>
<tr>
<td>DCIR</td>
<td>dendritic cell immunoreceptor</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>dendritic cell-specific ICAM-grabbing non-integrin</td>
</tr>
<tr>
<td>Dectin</td>
<td>DC-associated C-type lectin</td>
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<tr>
<td>DGDG</td>
<td>diglucosydialicglycerol</td>
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<tr>
<td>ds</td>
<td>double-stranded</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>Fc</td>
<td>fragment crystallizable</td>
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<td>FcR</td>
<td>Fc receptor</td>
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<tr>
<td>FcRγ</td>
<td>Fc receptor common gamma-chain</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAS</td>
<td>group A Streptococcus</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-acetylgalactosamine</td>
</tr>
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1
List of abbreviations

GBS  group B *Streptococcus*  
G-CSF  granulocyte-colony stimulating factor  
GFP  green fluorescence protein  
GI  gastrointestinal  
Glu  glutamate  
HIF-1  hypoxia-inducible factor-1  
HRP  horseradish peroxidase  
IFN  interferon  
Ig  immunoglobulin  
IL  interleukin  
IPS-1  interferon-beta promoter stimulator 1  
ITAM  immunoreceptor tyrosine-based activation motif  
ITIM  immunoreceptor tyrosine-based inhibitory motif  
LBP  lipopolysaccharide-binding protein  
LOS  lipooligosaccharide  
LPS  lipopolysaccharide  
LRR  leucine-rich repeat  
LTA  lipoteichoic acid anchor  
ManLAM  mannose-capped lipoarabinomannan  
MAPK  mitogen-activated protein kinases  
MCL  macrophage C-type lectin  
MDA5  melanoma differentiation-associated gene 5  
MDP  muramyl dipeptide  
MGDG  monoglucosyldiacylglycerol  
MGL  macrophage galactose-type C-type lectin  
MHC  major histocompatibility complex  
Mincle  macrophage-inducible C-type lectin  
MIP  macrophage inflammatory protein  
MR  mannose receptor  
MyD88  myeloid differentiation factor 88  
NFAT  nuclear factor of activated T cells  
NF-κB  nuclear factor kappa-light-chain-enhancer of activated B cells
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>NK cell</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOD</td>
<td>nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PHDs</td>
<td>prolyl hydroxylase domain enzymes</td>
</tr>
<tr>
<td>Pro</td>
<td>proline</td>
</tr>
<tr>
<td>Raf</td>
<td>rapidly accelerated fibrosarcoma</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated on activation normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RIG-I</td>
<td>retinoic acid inducible gene I</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-I-like receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>PRRs</td>
<td>pattern recognition receptors</td>
</tr>
<tr>
<td>SAP130</td>
<td>Sin3A associated protein 130</td>
</tr>
<tr>
<td>SIGNR</td>
<td>DC-SIGN-related protein</td>
</tr>
<tr>
<td>ss</td>
<td>single-stranded</td>
</tr>
<tr>
<td>STING</td>
<td>stimulator of interferon genes</td>
</tr>
<tr>
<td>Syk</td>
<td>spleen tyrosine kinase</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen processing</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TDB</td>
<td>trehalose-6,6-dibehenate</td>
</tr>
<tr>
<td>TDM</td>
<td>trehalose-6,6-dimycolate</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll-Interleukin 1 Receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
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<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-β</td>
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Sabine Mayer-Lambertz: Role of C-type lectin receptors in bacterial recognition

The most important function of the innate immune system is the recognition of pathogens that may cause damage to the host. To sense foreign structures, host immune cells express so-called pattern-recognition receptors (PRRs) on their surface to detect highly conserved pathogen-associated molecular patterns (PAMPs) present in all classes of pathogens like bacteria, viruses, parasites, and fungi. PRRs comprise soluble and transmembrane receptors, including intracellular receptors as nucleotide-binding oligomerization domain-like receptors, retinoic acid inducible gene I receptors and Toll-like receptors, but also the extracellularly located C-type lectin receptors (CLRs). CLRs were originally described as calcium-dependent carbohydrate-binding proteins. Nowadays, it is known that CLRs additionally detect other ligands than carbohydrates such as lipids, proteins, ice crystals and inorganic molecules. Furthermore, also calcium-independent ligand binding of CLRs has been described. Macrophage-inducible C-type lectin (Mincle) is a type II transmembrane protein expressed by monocytes, neutrophils, macrophages and dendritic cells. Activation of Mincle by ligand recognition leads to phosphorylation of spleen-tyrosine kinase that in turn induces the formation of the ternary CARD9/Bcl10/Malt1 complex and the activation of the transcription factor NFκ-B. Finally, NFκ-B triggers effector functions such as the production of cyto- and chemokines, cell degranulation and phagocytosis. Bacteria are known to express various carbohydrate structures (glycans) on their surface that play important roles in host cell adhesion, invasion and immune evasion. Since these structures are crucial for bacterial colonization, survival, growth and initiation of pathogenesis, there is rising interest in detecting novel CLR/bacteria interactions to prevent infections.

Chapter 3 of this thesis deals with the detailed description of three different methodologies to detect yet unknown CLR/bacteria interactions using the very frequent human pathogen *Campylobacter jejuni* as model organism. All methods presented can be easily applied to other Gram-negative and Gram-positive bacteria and build on a comprehensive CLR-hFc fusion protein library. CLR-hFc fusion proteins consist of the Fc part of a human IgG1 fused to the carbohydrate-recognition domain (CRD) of the respective CLR. First, an ELISA-based high-throughput assay is described that allows for an initial pre-screening for CLR/bacteria interactions. This method relies on plate-coated bacteria that are incubated with the respective
Abstract

fusion proteins. Finally, binding is detected by reaction of the colorimetric substrate ABTS with a secondary anti-human IgG1 Fc antibody coupled to horseradish peroxidase. Among others, Dectin-1-hFc was found to bind to C. jejuni isolates MHH19 and MHH24. Since false-positive CLR candidates may occur due to protein aggregation, a following flow cytometry-based assay was established to confirm potential binding partners. Here, bacteria were kept in solution, incubated with the CLR-hFc fusion proteins and binding was detected by the usage of an anti-human IgG1-Fc antibody in a subsequent flow cytometric analysis. Applying this protocol, binding to Dectin-1-hFc was confirmed for both C. jejuni isolates. Finally, a confocal microscopy approach is presented that enables direct visualization of the CLR binding to bacteria. Also, in this assay, Dectin-1-hFc was found to recognise both C. jejuni isolates. In summary, the described methods based on CLR-hFc fusion proteins offer the possibility to screen for various novel CLR/bacteria interactions. The detection of these candidates presents the first step towards bacterial ligand identification and the analysis of the biological function of the respective CLRs.

The identification of Mincle as CLR that recognises parts of the lipoteichoic acid anchor (LTA) present in the cell wall of the human pathogen group A Streptococcus (GAS) and thereby promoting antibacterial immunity is part of chapter 4. Gene expression analysis and cell stimulation assays revealed that the Mincle adaptor protein CARD9 is involved in the immune responses against GAS infection. Stimulation of bone marrow-derived dendritic cells (BMDCs) with purified monoglucosyldiacylglycerol (MGDG) of LTA led to a Mincle-dependent production of inflammatory cytokines, reactive oxygen species (ROS) and the activation of inducible nitric oxide synthase (iNOS). In addition, the purified diglucosyldiacylglycerol (DGDG) of the LTA is recognised by Mincle but does not initiate signaling. Furthermore, it was found that DGDG serves as suppressor of Mincle whereas MGDG mediates agonistic Mincle activation. In vivo, Mincle-deficient mice infected with GAS strains others than the 5448 exhibited high mortality in line with severe bacteraemia and an increased expression of proinflammatory cytokines. In summary, these results suggest a protective role for Mincle in anti-GAS immunity.

This work presents comprehensive methods to detect novel CLR/bacteria interactions and contributed to the identification of GAS-derived MGDG and DGDG as new Mincle ligands.
Zusammenfassung

Sabine Mayer-Lambertz: Die Rolle von C-Typ Lektin Rezeptoren in der Erkennung von Bakterien


Das Kapitel 3 beschäftigt sich mit drei unterschiedlichen Methoden, um bislang unbekannte CLR/Bakterien-Interaktionen aufzudecken. Dabei werden die Methoden anhand des
Zusammenfassung


Das Kapitel 4 dieser These beschreibt die Identifikation von Strukturen des Lipoteichonsäureankers (LTA) in Gruppe A Streptokokken (GAS) als Mincle-Liganden, die in der Lage sind, eine Immunantwort gegen GAS auszulösen. Genexpressionsstudien und Zellstimulationsexperimente zeigten, dass CARD9, ein Adaptermolekül von Mincle, an der Immunantwort gegen GAS beteiligt ist. Die Stimulation von Makrophagen, die aus dem Knochenmark differenziert wurden, mit aufgereinigtem Monoglycosyldiacylglycerol (MGDG) aus dem LTA führte zu einer Mincle-abhängigen Produktion von proinflammatorischen Zytokinen, reaktiven Sauerstoffspezies (ROS) und der Aktivierung der Stickstoffmonoxid-Synthase (iNOS). Zusätzlich wurde gezeigt, dass von dem LTA aufgereinigtes Diglycosyldiacylglycerol (DGDG) von Mincle erkannt wird, jedoch zu keiner
Zusammenfassung


Zusammenfassend präsentiert diese Arbeit umfangreiche Methoden, um neue Interaktionen von CLR und Bakterien zu detektieren. Außerdem trägt diese Arbeit zu der Entdeckung bei, dass die beiden LTA Bestandteile MGDG und DGDG aus GAS als neue Mincle Liganden fungieren.
Chapter 1: General introduction

1. The immune system - essential for survival

“I find it astonishing that the immune system embodies a degree of complexity which suggests some more or less superficial though striking analogies with human language, and that this cognitive system has evolved and functions without assistance of the brain” -Niels K. Jerne-

The human body is challenged every day by multifaceted microorganisms, like fungi, viruses, parasites and bacteria that attempt to invade and colonize the host. In addition to these environmental pathogens, the body is naturally colonized by approximately \(10^{14}\) microorganisms that harbor due to dysbiosis an increased risk for causing disease to the host (1, 2). Nevertheless, it is the result of the immune system as a powerful defense system that the host is usually able to limit the infection and eliminate pathogens fast. The immune system (lat.: immunis; free from, devoid of) is one of the most complex systems in the human body, forming a remarkable network of different tissues, cells and proteins that cooperate to protect the body from foreign structures such as microbes, viruses, cancer cells and toxins (3). The immune system is composed of two potent branches, the innate and the adaptive immune system (figure 1). Whereas for fast and effective protection of the host both parts have to collaborate using humoral and cellular components, each part clearly mediates distinct functions. The innate immune system initiates the first responses after the host is exposed to pathogens. To allow a fast detection of and response to invading pathogens, the innate immune system provides various genetically predefined receptors, so-called pattern-recognition receptors (PRRs), that recognize evolutionarily highly conserved microbial structures on a broad spectrum of pathogens (Pathogen-associated molecular patterns, PAMPs) or frequent signs of infections (Danger-associated molecular patterns, DAMPs). However, this only induces a general immune response that includes the induction of inflammatory responses, the process of tissue repair, the recruitment of immune cells and the release of cytokines and chemokines. These processes are activated directly after the infection occurred and they are not influenced in strength by repeated exposure to the same antigen (3-5). In contrast, dependent on the activation by the innate immune system, the adaptive immune system relies on a defined repertoire of specific receptors that enables response to any antigen (6). Since the development of these highly specific components requests time, the adaptive immune system is only activated after several days of infection. The adaptive immune system displays memory, thereby providing an augmented immune
reaction towards antigens that had already been encountered previously by the host (4, 7). However, any disturbed immune function in parts of the immune system increases the susceptibility of the host to infections, highlighting the importance of an efficient interaction of the innate and adaptive immune system (3).

1.1 Innate Immunity

The innate immune system is the crucial first line of defence against infections and includes the epithelium as the initial and main barrier against pathogens. The epithelium is located in the respiratory tract, skin and gastrointestinal (GI) tract; hence, it is the part that is challenged most frequently with invading pathogens. Thus, it offers multifaceted strategies to prevent microbial entry and spread. Tight junctions connect cells with each other and provide an important physical barrier (8) whereas mucus produced by the respiratory and GI tract enables rapid entrapment of microbes and protects the epithelial surface. In addition, physical and chemical barriers such as temperature, low pH or the secretion of antimicrobial substances kill or neutralize pathogens (4, 9, 10). Importantly, tissue cells that encountered pathogens produce cytokines that further activate innate immune cells and guide them to the site of infection (11). The innate response also includes a soluble fraction that comprises acute-phase proteins, interferons (IFNs), antimicrobial peptides and the complement system (figure 1). Most notably, the complement system consists of more than 30 plasma proteins that are cascade-like activated to identify, opsonize and kill pathogens or infected cells either directly or by phagocytosis. Furthermore, it activates and mobilizes side by side with released cytokines the cellular component of the innate immune system that comprises monocytes, granulocytes, macrophages, mast cells, dendritic cells (DCs) and natural killer (NK) cells (figure 1) (3, 12).

Of these cell subsets, DCs and macrophages are termed professional antigen-presenting cells (APCs) since they are able to present antigens to T cells in the presence of co-stimulatory molecules (figure 1), hence linking innate with adaptive immunity. Besides that, macrophages are characterised by their pronounced phagocytic ability. If the epithelial barrier is breached by physical damage and pathogens can invade the host, macrophages incorporate intruders into large intracellular organelles, so-called phagosomes. This compartment fuses with a lysosome containing enzymes, peptides and proteins to a phagolysosome and subsequently leads to the degradation of the internal content (10, 13). In addition to the pure
elimination of pathogens, peptide fragments can be presented by macrophages on major histocompatibility complex (MHC) II molecules to T cells, leading to their activation. In addition, macrophages initiate a potent response towards intracellular pathogens via the IFNγ-mediated activation by Th1-cells (3, 4).

Whereas macrophages are defined by their phagocytic activity, the primary function of DCs is the activation of the adaptive immune system by the presentation of antigens to T cells. Immature DCs are resident throughout the tissues where they capture antigens with surface located receptors, e.g. CLRs, but also via Fc receptors (FcRs) (14, 15). The processing of respective antigens into proteolytic peptides is mediated in the next step via phagocytosis, macropinocytosis or receptor-mediated endocytosis and leads to the maturation of DCs (16). DCs take up larger organisms such as parasites or fungi and they are able to ingest various Gram-positive and Gram-negative bacteria as well as mycobacteria (17). The uptake of *Salmonella* by the gut epithelium is directly mediated by DCs that open tight junctions to expose their dendrites to directly capture bacteria (18). In addition, DCs are often the target of virus infections. Surface molecules on DCs enable viruses to enter the cells and start the production of virus-associated particles in the cytoplasm. To ensure an effective immune response towards these various extra- and intracellular stimuli, DCs express two different classes of MHC molecules that trigger different immune response pathways. Whereas endogenous (intracellular) peptides presented on MHC I molecules activate CD8⁺ cytotoxic T lymphocytes (CTLs), MHC II molecules present exogenous peptides to CD4⁺ T cells (6, 19). In addition to this classical antigen-presentation pathway, the mechanism of cross-presentation enables presentation of extracellular peptides to naïve CD8⁺ T cells to become activated CD8⁺ CTLs. This pathway is especially important for the initiation of immune responses towards peripheral tissue cells others than antigen-presenting cells infected with viruses or even against tumor cells (20).

### 1.2 Adaptive Immunity

Whereas more primitive organisms like invertebrates and plants exclusively rely on the innate immune system, higher animals further developed the adaptive immune system: a clonal- and antibody-based second branch that enables immunological memory, hence increasing the reaction towards reoccurring intruders (12, 21). In parallel to the innate immune system, the adaptive immune system is activated. The adaptive immune system is characterised by the
formation of pathogen-specific pathways. Hallmark cells of this branch are antigen-specific T cells and antibody-producing B cells that represent clonal lymphocyte populations (figure 1). Whereas all lymphocyte progenitor cells develop from hematopoietic stem cells in the bone marrow, where B cells persist to undergo maturation, immature T cells are subsequently recruited to the thymus to reach their final maturation state (7).

T cells are characterised by specific surface-expressed T cell receptors (TCRs). These TCRs recognize antigen fragments presented by APCs on MHC I or MHC II molecules. In addition to the TCR/peptide/MHC interaction, a binding of the costimulatory molecules CD80 or CD86 located on mature APCs to CD28 expressed on T cells is required to fully activate T cells (22). Upon recognition of the antigen bound to MHC-I, naïve CD8+ CTLs differentiate into mature CTLs. Once activated, mature CD8+ T cells release effector molecules, such as perforin and granzyme B, that induce the apoptosis of infected and abnormal cells (6). After successful elimination of foreign agents, only few cells survive as memory T cells that protect the host against reinfection due to their rapid response (23). The presentation of antigens bound to MHC-II molecules stimulates CD4+ T cells. These cells regulate in particular the type of immune response by the release of cytokines that enable an enhanced reaction to eliminate pathogens. Therefore, CD4+ T cells differentiate into various types of T helper (Th) cells (figure 1) (24). Most notably, Type 1 helper (Th1) cells mediate a cellular immune response by the release of IFNγ and interleukin (IL)-2, whereas a reaction initiated by Th2 cells is characterized by IL-4, IL-5, IL-10 and IL-13 production, that in turn efficiently activates naïve B cells to produce antibodies (humoral immune response) (6). Th17 cells are characterized by their ability to produce IL-17. IL-17A and IL-17F are known to be important proinflammatory cytokines that induce the production of IL6 and tumor necrosis factor α (TNFα) as well as the recruitment of granulocytes (6). Th9 cells play an important role in allergy and autoimmunity as well as in parasitic helminth infection (25) and tumor eradication (26). Additionally, regulatory T cells (Tregs), another CD4+ T cell subset, suppress or downregulate immune responses, thereby playing an important role in maintaining immune homeostasis (27).
Introduction

Figure 1: Components of the innate and adaptive immune system. Both branches of the immune system contain cellular and humoral components. The innate immune system resembles the first line of host defense and comprises phagocytic immune cells like macrophages as well as classical antigen-presenting cells (APCs) such as dendritic cells (DCs). The humoral part includes host defense peptides like antimicrobial peptides or the LPS Binding Protein. If the innate immune response is unable to clear the pathogens, the adaptive immune system is initiated after several hours. Crucial for the activation is the recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) by cellular receptors located on APCs, e.g., C-type lectin receptors and the subsequent presentation of peptide fragments loaded on major histocompatibility complexes (MHC) to T cells via T cell receptor (TCR)/MHC-interaction. Antigen presentation stimulates immature T cells to differentiate into cytotoxic T cells (CTL) or T helper (Th) cells. Activation of Th cells drives down one of several differentiation pathways. One subset represents the regulatory T cells (Tregs) which dampen immune activation. In contrast, Th1 response activates killing of intruders, Th2 cells are involved in antibody production and clearance of helminths whereas Th17 cells induce inflammatory responses. Th9 cells play a role in anti-tumor immunity, worm infections as well as in autoimmunity and allergy. (4)

B cells mediate an important role in the humoral part of the adaptive immune system. Each B cell expresses approximately 1.5x10^5 molecules of a unique antigen-binding receptor (BCR) on its surface that allows a direct recognition and highly specific binding of an antigen (2). Once activated by foreign antigens, naïve B cells divide rapidly by clonal expansion into plasma cells or into memory B cells. Plasma cells produce a large amount of respective
antibodies that in turn are able to bind to specific antigens on pathogens, hence flagging them for uptake by phagocytes. Whereas these plasma cells die after one to two weeks, memory B cells survive and continue the production of antibodies (28). Long-living memory T and B cells present important survivors of an overcome infection and B cells produce antibodies that allow immunological memory, enabling a faster and effective immune reaction towards previously encountered antigens.

2. Of PAMPs, DAMPS and PRRs

For a long time the adaptive immune system was seen as the more powerful arm of immunity. With the introduction of a model of microbial pattern recognition by Charles Janeway Jr., the innate immune system got more into the focus of many research groups. Janeway’s prediction relies on a limited number of germ line-encoded PRRs that have genetically determined specificities for various microbes and other foreign molecules (5) and are mainly present on APCs and other immune but also non-immune cells (29). PRRs recognize highly conserved structures called PAMPs that are abundantly expressed by all classes of pathogens including viruses, protozoa, fungi as well as Gram-positive and -negative bacteria (30). Since PAMPs such as LPS or bacterial CpG DNA are exclusively present in microbes and essential for pathogen survival and integrity, they represent suitable molecules for microbial sensing by host cells (5, 29). However, besides the recognition of external-derived PAMPs, PRRs also sense endogenous DAMPs. DAMPs represent host factors that are either released by the extracellular matrix (ECM) or from cells upon infection, inflammation or other types of cellular stress (31). Upon disruption of the cell, DAMPs that for example are under healthy conditions attached to the cytoskeleton or the ECM, such as actin (32) or fibrinogen (33), are proteolytically released into the extracellular space and are involved in inflammation and cell regeneration processes in a PRR-dependent manner (34). In addition to ECM-derived DAMPs, various soluble molecules are intracellularly generated or accessible during cellular necrosis or apoptosis. According to their origin within the cell, these molecules can be classified into different groups: mitochondria-derived DAMPs such as mitochondrial DNA (35); DAMPs like ATP (36, 37) and IL-1β (38) that are produced during the process of autophagy as well as nuclear (e.g. DNA (39), histones (40)) and cytosolic (e.g. uric acid (41)) DAMPs (34).
Introduction

Up to now, the receptor system consists of several large families of PRRs (figure 2) including Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) that are membrane-bound, hence they detect pathogens present in endocytic compartments or in the extracellular space. Additionally, unbound intracellular receptors such as NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and AIM2-like receptors (ALRs) are located in the cytoplasm, hence they are responsible for the detection of intracellular agents e.g. via the endoplasmic reticulum membrane-resident adaptor stimulator of interferon genes (STING) (42). Remarkably, this compartmentalization of the receptors provides important information on the class of detected pathogens as well as their location, thus indirectly indicating their potential damage to the host cell (43, 44). For example, it is known that the extracellular presence of *Salmonella* is detected in a TLR-dependent manner leading to proinflammatory cytokine production and phagocytosis whereas the intracellular detection of *Salmonella* leads to death of the respective cell, initiated by NLRs (45). Along that line, the recognition of self-derived nucleic acids is connected to multiple disorders (46, 47). Therefore, compartmentalization of TLRs and their ligands provides a mechanism that enables the receptor to distinguish between foreign and self-derived nucleic acids (48). For example, the expression of TLR9 in intracellular compartments prevents the recognition of self-derived nucleic acids but enables sensing of virus-derived DNA (49).

Stimulation of PRRs by ligand recognition leads to the activation of distinct adaptor molecules that in turn trigger specific intracellular signaling pathways. Adaptor proteins play a crucial role in signal integration from several receptors and enable the detection of various ligands. Finally, the initiation of an enzymatic signal activates immune responses including inflammatory pathways such as the stimulation of APCs by the production of proinflammatory cytokines and IFNs (43, 50). This further elicits the adaptive immune responses. In addition, the recruitment of PRRs also leads to direct events like autophagy (51), cell death (52) and phagocytosis (53). These mechanisms finally orchestrate to allow for a fast and efficient response towards the intruders (43).
Figure 2: Pattern-recognition receptors (PRRs) with individual family members and their ligands. Pathogen-associated molecular patterns (PAMPs) are detected by several PRRs located in the cytoplasm of the immune cells such as NOD-like receptors (NLRs) or RIG-I-like receptors (RLRs). Furthermore, members of the Toll-like receptor (TLRs) and C-type lectin receptor (CLRs) families are expressed as transmembrane receptors. This figure shows the main classes of PRRs including some members with their described ligands as examples. (54, 55)

2.1 TLRs: their discovery changed the understanding of pathogen sensing

TLRs are probably the most intensively investigated class of PRRs. The first receptor discovered was named Toll and mediates dorsoventral development during embryogenesis in the fruit fly Drosophila melanogaster (56). Later, it was detected that Toll additionally plays an important role in the protection of flies against fungal infections (57). Up to now, 11 human (TLR1-10) and 12 murine (TLR1-9 and TLR11-13) homologues of Toll are described (58). TLRs are type I transmembrane glycoproteins and are mostly expressed by APCs like macrophages, DCs, and B lymphocytes (48). TLRs are characterized by an extracellular leucine-rich domain (LRR) that mediates ligand binding and a Toll/IL-1 receptor domain (TIR) for signal transduction, hence located in the intracellular or cytoplasmic space (59). So far, TLR ligands that are recognized by the LRR domain are described for most of the TLRs as well as the role of TLRs in host response to various microbes such as viruses, bacteria,
fungi and parasites (50, 60-64). PAMP binding to the respective TLR induces receptor oligomerization followed by the induction of intracellular signaling that generates a potent antimicrobial proinflammatory response (65). For most of the TLRs, except for TLR3, the recruitment of the adaptor protein myeloid differentiation primary response gene 88 (MyD88) is essential for induction of downstream signaling cascades (66). TLR3 in contrast interacts with Toll/IL-1R-domain-containing adaptor-inducing IFN-β (TRIF) that together with a bridging molecule is also used by TLR4. The engagement of various downstream adaptors involved in signal transduction finally leads to the translocation of the nuclear factor kappa B (NFκ-B) to the nucleus where it induces subsequently the expression of target genes involved in inflammatory responses towards intruders (2, 54).

TLRs are classified according to the nature of their PAMP ligand into two subclasses. Whereas TLR1, TLR2, TLR4, TLR5 and TLR6 recognise lipoproteins and lipoglycans, TLR3, TLR7, TLR8 and TLR9 are involved in the detection of nucleic acids (50). Focusing on bacterial TLR ligands, the interaction of TLR4 and bacterial lipopolysaccharide (LPS) that is ubiquitously present in the cell wall of Gram-negative bacteria has been described most intensively (67, 68). Since a TLR4/LPS interaction alone only leads to insufficient cell signaling, the accessory proteins LPS-Binding Protein (LBP) and MD2 assist in LPS binding and recognition by TLR4 (69, 70). In addition to many bacterial cell wall components that serve as PAMPs for TLRs including lipoarabinomannan (TLR2 (71)), flagellin (TLR5 (72)) and lipoteichoic acid anchor (LTA, TLR2 (73-75), TLR2/TLR6 (76)), also bacteria-derived DNA that contains hypomethylated CpG domains is recognized (TLR9 (77)). Furthermore, TLRs are divided according to their localization within the cell. TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed at the cell surface whereas the nucleic acid sensing TLRs (TLR3, TLR7, TLR8, TLR9) are located in membranes of the endosomal compartment (48). To enable sensing of intracellular cytosolic pathogen-derived PAMPs, like viral double-stranded (ds)RNA or single-stranded (ss)RNA, cells express additionally a large repertoire of cytosolic PRRs including RLRs and NLRs (65).

2.2 NLRs and RLRs: soluble cytoplasmic sensors of PAMPs and DAMPs

NLRs are a group of PRRs that have gained rising interest in the last years since all described members are connected to certain human diseases. NLRs are important cytosolic sensors of mainly bacteria-derived molecules present in the cytoplasm but also of environmental
components and host cell molecules (50). Examples of ligands derived from bacteria are: pore-forming toxins, flagellin proteins, muramyl dipeptide (MDP), anthrax lethal toxin or microbial RNA and DNA (78). In general, NLRs are characterized by multiple LRRs that mediate ligand recognition, a nucleotide-binding oligomerization domain (NOD) responsible for oligomerization upon ligand binding and a protein-protein interaction domain, that is specific for each NLR (79). Up to now, 22 NLRs are described that, based on their N-terminal part, are divided into 4 subfamilies including CIITA, NLRB, NLRC and NLRP (80). Additionally, NLRs can also be classified by their effector functions that they initiate upon ligand binding (81). Accordingly, some members of NLRs including NLRP1, NLRP3 and NLRC4 form a signaling complex, the inflammasome, that has a pivotal role in inflammatory cell death as well as processing and maturation of the proinflammatory cytokines IL-1β and IL-18 (82). Moreover, activated NLRs like NOD1 and NOD2 affect signal transduction via downstream adaptor molecules that lead to the activation of NFκ-B or activator protein 1 (AP1), hence enhancing the transcription of various proinflammatory cytokines (83, 84). In addition to the initiation of signal transduction, NLRs such as CIITA and NLRC5 are described to induce with the transcription of MHC-II and I genes, respectively, as the lack of MHC-II expression in patients with bare lymphocyte syndrome can be completely compensated by CIITA (85-87). Furthermore, NLRs are also involved in the process of autophagy, a fundamental cellular process of antigen elimination. NOD1 and NOD2 are known to induce autophagy to remove bacterial components (88).

Whereas NLRs represent soluble receptors that recognise various cytoplasmic pathogens, RLRs play a pivotal role in the detection and defense against viruses. Up to now, the family consists of three members: RIG-I, MDA5 and LGP2 which commonly recognize RNA viruses and subsequently lead to the induction of type I IFN, hence eliciting antiviral response (54). MDA5 recognizes picornaviruses as encephalomyocarditis virus whereas RIG-I is described to recognize several ssRNA viruses like Sendai virus, paramyxovirus and influenza virus (89, 90). The detection of the 5’-triphosphate present in virus ssRNA enables a selective discrimination of viral RNA from host RNA by RIG-I (91). Ligand recognition by RIG-I or MDA5 leads to a complex downstream signaling that involves several signaling adaptor proteins such as IFN-β promotor stimulator 1 (IPS-1) (92). Finally, the activated signal cascades result in the expression of type I IFNs and proinflammatory factors that are involved in efficient antiviral defense (54, 93).
2.3 CLRs: A diverse group that recognises sweet PAMPs

CLRs represent a highly diverse group of promiscuous receptors that were originally described to show a strong affinity for mannose, fucose and glucan carbohydrate structures present in all classes of pathogens, including viruses, parasites, yeast, fungi as well as Gram-positive and Gram-negative bacteria (94). In general, the group of CLRs comprises both, soluble and cell membrane-bound proteins (95). CLRs are characterized by the expression of one or more carbohydrate recognition domains (CRDs) or a C-type lectin-like domain (CTLD). Classically, CRD/carbohydrate binding is mediated in a Ca\(^{2+}\)-dependent manner as Ca\(^{2+}\)-ions are essential for ligand complexation and receptor integrity, hence determining its activity. CTLDs are also present in CLRs that recognize other PAMPs than carbohydrates such as proteins, crystals or lipid moieties. In addition, ligand recognition by these CLRs is mainly mediated in a Ca\(^{2+}\)-independent way (96, 97). Based on several criteria like phylogeny and structure, vertebrate CLRs are divided into 17 groups (98). Commonly, CLRs of the myeloid group are structured into an extracellular part with the CRD or CTL, a transmembrane segment and a cytoplasmic domain that in most cases harbors the signaling motifs of the respective CLRs. In the immune system, CLRs elicit various diverse functions like cell-cell adhesion, signal transduction and turnover of plasma glycoproteins (99). Nevertheless, one of the main function of CLRs is the recognition and subsequent uptake of pathogens. After being internalized, pathogens are degraded in lysosomes and their fragments are presented on MHC molecules by APCs to induce an adaptive immune response (100). Due to the large number of members within the CLR superfamily, the following section focuses on the subfamily of myeloid CLRs that consists of members with a pivotal role in PAMP detection and subsequent modulation of immune responses.

These CLRs are expressed by myeloid cells, especially APCs like macrophages, DCs and B cells and are involved in pathogen binding, uptake and degradation, thus playing a role in activation and regulation of antimicrobial responses (101). Moreover, CLRs are subclassified by their regulatory effect on the outcome of the initiated immune response. Accordingly, CLRs such as Dectin-2, macrophage-inducible C-type lectin (Mincle) (figure 3) and DCAR are associated with adaptor molecules (e.g. Fc Receptor common gamma-chain (FcR\(\gamma\))) containing immunoreceptor tyrosine-based activation motif (ITAM, YxxL/I) domains that activate downstream signaling pathways. Alternatively, CLRs like Dectin-1, SIGNR3 and...
Clec9a carry a single ITAM motif called hemITAM in their intracellular domain (102-104). Upon ligand detection, ITAM- and also to a certain extent hemITAM bearing CLRs (105, 106) induce cell signaling by recruitment of spleen tyrosine kinase (Syk), that in turn leads to the phosphorylation of tyrosines and activation of various intermediate molecules, including the activation of transcriptional pathways such as mitogen-activated protein kinases (MAPK) (107) and nuclear factor of activated T cells (NFAT) (102, 108). In addition, signaling via Syk activates the NLRP3 inflammasome that induces the maturation and secretion of IL-1\(\beta\), hence playing a role in antifungal host defence (109-112). Essentially, Syk signaling leads to the formation of an adaptor complex of Caspase recruitment domain-containing 9 (CARD9), B cell lymphoma 10 (Bcl10) and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (Malt1) that triggers proinflammatory gene expression via activation of the NF\(\kappa\)B pathway (figure 3) (113, 114). CLRs with immunoreceptor tyrosine-based inhibitory motif (ITIM, S/I/V/LxYxxI/V/L) domains mediate suppression of effector functions by activation of tyrosine phosphatases (SHP-1, SHP-2, SHIP). This group comprises CLRs such as Clec12a, DCIR and Clec12b. In addition, CLRs including Mannose-receptor (MR), SIGNR1 and DC-SIGN use complex signaling pathways independent of an ITAM or ITIM motif (115). However, some CLRs are able to trigger a Syk-independent activation of the NF\(\kappa\)B pathway by recruitment of the serine/threonine kinase Raf1 (116). Finally, activation of CLRs by ligand binding induces various cell responses like endocytosis, phagocytosis, production of chemokines and cytokines, complement activation and induction of the adaptive immune system (101).

Since pathogens express multiple PAMPs that can be recognized by several receptors, some CLRs perform crosstalk with different PRRs, including TLRs to enable a response concurrently against a broad range of pathogens (117). For example, it is well known that only the cooperation of DC-SIGN with TLRs triggers a cellular response whereas DC-SIGN signaling alone does not stimulate cytokine production in general, hence it rather modulates TLR-mediated responses (118, 119). In addition to crosstalk with other PRRs than CLRs, the formation of CLR-derived homo- or heterocomplexes, termed multimerization, affects receptor signaling and effector functions. Especially CLRs with hemITAMs like Clec2 tend to form homodimers since the successful activation of Syk requires two phosphorylated tyrosines (120, 121). Moreover, DC-SIGN, a CLR that lacks signaling motifs, forms tetramers that increases receptor avidity and specificity (122). However, the engagement of
CLRs like Mincle (figure 3), Dectin-1 and Dectin-2 induces various immune responses upon pathogen detection that are independent of other PRRs.

Figure 3: CLR engagement triggers various immune responses. This is displayed in this graphic for Mincle as example. The Mincle carbohydrate-recognition domain (CRD) senses diverse glycan structures from several pathogens like fungi and bacteria. Additionally, also danger-associated molecules released by damaged or dead cells activate Mincle. Upon activation, the spleen-tyrosine kinase (Syk) is recruited via the ITAM-bearing FcRγ stalk which leads to the formation of the CARD9/Bcl10/Malt1 complex and further to the activation of the transcription factor NFκB. The subsequent production of cytokines and the activation of other effector functions initiate important mechanisms involved in host defense, tissue repair mechanisms and autoimmune diseases. (123)

The following section will provide a comprehensive introduction into the role of CLRs in anti-bacterial immunity with a focus on the CARD9-dependent CLRs Mincle and Dectin-1. These CLRs were identified to play an important role in bacterial recognition during the projects that are included in this thesis.

2.3.1 Role of CLRs in anti-bacterial immunity

Bacteria express various virulence factors such as adhesins, toxins and enzymes that contribute to pathogenesis in the host. Carbohydrate-based structures such as the M-protein, lipoteichoic acid anchor (LTA), capsular polysaccharide (CPS) or O-antigen domains derived from bacterial lipopolyssacharides (LPS) facilitate bacteria/host interactions (124-128).
Hence, these structures are essential for adherence and subsequent colonization of host tissue by bacteria, thus mediating the first and most important step in establishment of an infection. Since bacterial glycosylation is usually the first “pattern” encountered by cells of the host immune system, there is growing interest on the role of CLRs in bacterial recognition. The impact of CLRs is best described for *Mycobacterium tuberculosis*. Up to now, several CLRs including MR (129), DC-SIGN (130), Mincle (131, 132) and Dectin-1 (133) have been described to interact with *M. tuberculosis* (134). However, whereas several reports on CLR/*M. tuberculosis* interactions are described *in vitro*, the respective receptors showed only limited to redundant role in clearance of the infection *in vivo* (130, 135, 136). MR was shown to recognise mannose-capped lipoarabinomannan (ManLAM) present on the surface of *M. tuberculosis* and affected the phagocytosis and assembly of the phagolysosome (129, 137). Additionally, it is reported that the tetrameric transmembrane protein DC-SIGN recognises mannose moieties in ManLAM structures of mycobacteria and influences the expression of costimulatory molecules and immune responses in human macrophages (138). Although several murine homologs of DC-SIGN exist, SIGNR3 has been identified as the closest functional orthologue of human DC-SIGN based on binding studies (139, 140). Infection of SIGNR3-deficient mice with *M. tuberculosis* led to increased bacterial load in the early phase of infection. However, in the later course of infection, these mice efficiently exhibited an adaptive immune response, comparable to wild-type mice (130). Besides *M. tuberculosis*, DC-SIGN is also described to interact with other bacteria like *Helicobacter pylori* and a number of *Lactobacillus* strains (94). Here, the surface layer A protein of *L. acidophilus* was detected as ligand for DC-SIGN (141).

Although contradictory reports on the role of Mincle in murine infection studies with different bacterial species exist, its adaptor signaling protein CARD9 mediates an essential role in defense against *Mycobacterium* species, since CARD9-deficient mice showed exacerbated pathogenesis and rapid death in an infection study *in vivo* (142). The mycobacterial cell wall glycolipid trehalose-6,6-dimycolate (cord factor, TDM) is a well-known ligand for the CLR Mincle, capable of initiating an inflammatory response including granuloma formation in mice (131, 132). In addition to TDM, Mincle also recognises the synthetic analog trehalose-6,6-dibehenate (TDB) (131). The crystal structure of the recognition sites of bovine and human Mincle binding to trehalose unraveled a canonical C-type primary monosaccaride-binding site that complexes a Ca²⁺ ion (143, 144). Moreover, a
primary and a proximal secondary binding site complex both glucose moieties in the head group of the trehalose whereas a hydrophobic groove opposite of the primary binding side was shown to sense fatty acids with a minimum of 10 carbon atoms (131, 143, 145). Interestingly, the design of mono- and diacetylated derivatives of trehalose with different numbers of attached acyl chains resulted in an increased affinity for Mincle binding with rising number of carbon atoms (146) (for further structural information about Mincle, see (147)). Mincle is a typ II transmembrane receptor that is mainly expressed by myeloid cells like macrophages, DCs, monocytes and neutrophils (148-152). However, the receptor was also found to be present on B cells (153). Activation of Mincle upon ligand binding to the CRD initiates the phosphorylation of the ITAM motif present in the FcRγ receptor associated to Mincle. Subsequently, the recruitment of Syk leads to the formation of the CARD9/Bcl10/Malt1 complex that activates NFκB-signaling pathway resulting in the expression of chemokines, proinflammatory cytokines such as IL-12 and TNF-α, induction of phagocytosis and nitric oxide (NO) production (figure 3) (148, 154, 155). Whereas most of the effector functions are proinflammatory, Mincle also induces the production of IL-10, hence the activation of anti-inflammatory responses, thereby being involved in both, immune homeostasis and immune modulation towards microbial pathogens (156). Mincle is described to interact with a number of diverse bacterial strains, like Streptococcus pneumoniae (157, 158), Klebsiella pneumoniae (159), H. pylori (160) and several Corynebacterium (161) and Lactobacillus strains (162, 163), but the actual Mincle ligand for most of the interactions in still unknown. Recently, it was shown that glucosylacylglycerol from the important human pathogens S. pneumoniae and group A Streptococcus (GAS, S. pyogenes) is recognised by Mincle (157, 164). Glucosylacylglycerol is part of the LTA that is the major component of the cell wall of Gram-positive bacteria. Besides binding of Mincle to the respective bacteria, it was additionally demonstrated that the lack of Mincle in mice infected with the respective bacteria resulted in increased mortality and dysregulated anti-bacterial immunity, hence pinpointing to a protective role of Mincle (157, 164). Aside from sensing bacteria, Mincle furthermore detects self-molecules such as SAP-130 (155) or β-glucosylceramide that derived from damaged or necrotic cells (165) and pathogens like parasites (Leishmania major) and fungi (101).

In fungi, the myeloid cell-derived CLR Dectin-1 is known to be the major receptor for β-1,3-linked glucan, an ubiquitous cell wall component of fungi (166, 167). Hence, its role in
sensing fungal pathogens such as *Pneumocystis carinii* (168), *Candida albicans* (168, 169), *Aspergillus fumigatus* (170-172) and *Cryptococcus neoformans* (173) has been intensively studied (174). Upon ligand detection by the single CTLD, Dectin-1 recruits Syk via phosphorylation of the hemITAM motif present in the cytoplasmic tail, followed by the engagement of the CARD9/Bcl10/Malt1 complex and subsequent activation of the NFκ-B pathway with the production of inflammatory cytokines and induction of other effector functions (175). Although several studies report a role for Dectin-1 in anti-mycobacterial defense *in vitro*, its role *in vivo* as well as the definite ligand present in mycobacteria still remains unclear since Dectin-1-deficient mice challenged with *M. tuberculosis* showed no significant difference in pathology and induced immune responses compared to wild-type mice (176). Moreover, Dectin-1 crosstalk with TLR2 is required for efficient response including phagocytosis, inflammatory cytokine production and induction of the MAPK pathways towards mycobacterial species *in vitro* (177).
3. Aims of the study

CLRs play an important role in pathogen detection and the subsequent initiation and modulation of various immune responses. Many interactions of CLRs and several pathogens like viruses, bacteria, parasites and fungi are described. Bacteria present interesting study targets since they express large numbers of glycoconjugates on their surface. In the field of microbiology, the role of CLRs is best described for *M. tuberculosis*, whereas for other bacteria the impact of CLRs still has to be elucidated more extensively. Importantly, some bacteria such as *S. pneumoniae* or *H. pylori* are shown to interact with CLRs but for most of the bacteria, distinct ligands of the respective receptors are still unknown. Moreover, targeting specific CLRs presents an interesting strategy to deliver drugs or vaccine antigens into specific cells to selectively modulate immune responses. Unraveling new CLR/bacteria interactions presents the first step towards identification of new CLR ligands and to get better insights into the biological relevance of the respective receptor.

**Aim 1: Establishment of different in vitro methods to detect novel CLR/bacteria interactions**

Based on the usage of a comprehensive CLR-hFc fusion protein library (178) and *Campylobacte jejuni* as representative model for various other bacteria, diverse methods have been established to enable screening for novel CLR/bacteria interactions. First, an ELISA-based assay allowed for high-throughput screening of plate-bound bacteria. Second, a flow cytometric assay has been established to verify potential CLR binding candidates and finally, the binding specificity of the respective CLR has been visualized using a confocal microscopy-based method. The combination of these three distinct methods should allow for identification of new CLR interactions with bacterial ligands, hence paving the way for further functional studies.

**Aim 2: Unraveling the role of the CLR Mincle in the recognition of group A Streptococcus**

GAS is an important human pathogen that can cause various diseases, ranging from self-limiting infections like pharyngitis or impetigo to severe outcomes such as necrotizing fasciitis or streptococcal toxic shock syndrome (179). Since several studies suggest that next
Aims of the study

To TLRs also other PRRs play an important role in the detection of GAS (180, 181), it was investigated if CLR may play a role in sensing GAS. In a collaborative approach, transcriptome analysis and cell culture-based methods like stimulation of bone marrow-derived DCs (BMDCs) and bone marrow-derived macrophages (BMMs) were applied to first identify a novel CLR that interacts with GAS, followed by analysis of the antibacterial response initiated by this CLR. Finally, the in vivo role of the respective CLR was analysed by murine infection studies using the respective CLR-deficient mice.
Chapter 2: Paper I

C-type lectins: their network and roles in pathogen recognition and immunity


Title: C-type lectins: their network and roles in pathogen recognition and immunity

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The extent of Sabine Mayer-Lambertz’s contribution to the article is evaluated according to the following scale:

A. has contributed to collaboration (0-33%).
B. has contributed significantly (34-66%).
C. has essentially performed this study independently (67-100%).

1. Design of the project including design of individual experiments: /
2. Performing of the experimental part of the study: /
3. Analysis of the experiments: /
4. Presentation and discussion of the study in article form: B
Abstract

C-type lectins (CTLs) represent the most complex family of animal/human lectins that comprises 17 different groups. During evolution, CTLs have developed by diversification to cover a broad range of glycan ligands. However, ligand binding by CTLs is not necessarily restricted to glycans as some CTLs also bind to proteins, lipids, inorganic molecules, or ice crystals. CTLs share a common fold that harbors a Ca2+ for contact to the sugar and about 18 invariant residues in a phylogenetically conserved pattern. In vertebrates, CTLs have numerous functions, including serum glycoprotein homeostasis, pathogen sensing, and the initiation of immune responses. Myeloid CTLs in innate immunity are mainly expressed by antigen-presenting cells and play a prominent role in the recognition of a variety of pathogens such as fungi, bacteria, viruses, and parasites. However, myeloid CTLs such as the macrophage inducible CTL (Mincle) or Clec-9a may also bind to self-antigens and thus contribute to immune homeostasis. While some CTLs induce pro-inflammatory responses and thereby lead to activation of adaptive immune responses, other CTLs act as inhibitory receptors and dampen cellular functions. Since CTLs are key players in pathogen recognition and innate immunity, targeting CTLs may be a promising strategy for cell-specific delivery of drugs or vaccine antigens and to modulate immune responses.
Chapter 3: Paper II

C-type lectin receptor (CLR)-Fc fusion proteins as tools to screen for novel CLR/bacteria interactions: an exemplary study on preselected *Campylobacter jejuni* isolates


Title: C-type lectin receptor (CLR)-Fc fusion proteins as tools to screen for novel CLR/bacteria interactions: an exemplary study on preselected *Campylobacter jejuni* isolates

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C. has essentially performed this study independently (67-100%).

1. Design of the project including design of individual experiments: B
2. Performing of the experimental part of the study: C
3. Analysis of the experiments: C
4. Presentation and discussion of the study in article form: B
Abstract

C-type lectin receptors (CLRs) are carbohydrate-binding receptors that recognize their ligands often in a Ca2+-dependent manner. Upon ligand binding, myeloid CLRs in innate immunity trigger or inhibit a variety of signaling pathways, thus initiating or modulating effector functions such as cytokine production, phagocytosis, and antigen presentation. CLRs bind to various pathogens, including viruses, fungi, parasites, and bacteria. The bacterium *Campylobacter jejuni* (*C. jejuni*) is a very frequent Gram-negative zoonotic pathogen of humans, causing severe intestinal symptoms. Interestingly, *C. jejuni* expresses several glycosylated surface structures, for example, the capsular polysaccharide (CPS), lipooligosaccharide (LOS), and envelope proteins. This “Methods” paper describes applications of CLR–Fc fusion proteins to screen for yet unknown CLR/bacteria interactions using *C. jejuni* as an example. ELISA-based detection of CLR/bacteria interactions allows a first prescreening that is further confirmed by flow cytometry-based binding analysis and visualized using confocal microscopy. By applying these methods, we identified Dectin-1 as a novel CLR recognizing two selected *C. jejuni* isolates with different LOS and CPS genotypes. In conclusion, the here-described applications of CLR–Fc fusion proteins represent useful methods to screen for and identify novel CLR/bacteria interactions.
Chapter 4: Paper III

Lipoteichoic acid anchor triggers Mincle to drive protective immunity against invasive group A *Streptococcus* infection


**Lipoteichoic acid anchor triggers Mincle to drive protective immunity against invasive group A *Streptococcus* infection**

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The extent of Sabine Mayer-Lambertz’s contribution to the article is evaluated according to the following scale:

A. has contributed to collaboration (0-33%).
B. has contributed significantly (34-66%).
C. has essentially performed this study independently (67-100%).

1. Design of the project including design of individual experiments: A
2. Performing of the experimental part of the study: A
3. Analysis of the experiments: A
4. Presentation and discussion of the study in article form: A
Abstract

Group A Streptococcus (GAS) is a Gram-positive bacterial pathogen that causes a range of diseases, including fatal invasive infections. However, the mechanisms by which the innate immune system recognizes GAS are not well understood. We herein report that the C-type lectin receptor macrophage inducible C-type lectin (Mincle) recognizes GAS and initiates antibacterial immunity. Gene expression analysis of myeloid cells upon GAS stimulation revealed the contribution of the caspase recruitment domain-containing protein 9 (CARD9) pathway to the antibacterial responses. Among receptors signaling through CARD9, Mincle induced the production of inflammatory cytokines, inducible nitric oxide synthase, and reactive oxygen species upon recognition of the anchor of lipoteichoic acid, monoglycosyldiacylglycerol (MGDG), produced by GAS. Upon GAS infection, Mincle-deficient mice exhibited impaired production of proinflammatory cytokines, severe bacteremia, and rapid lethality. GAS also possesses another Mincle ligand, diglycosyldiacylglycerol; however, this glycolipid interfered with MGDG-induced activation. These results indicate that Mincle plays a central role in protective immunity against acute GAS infection.
Chapter 5: Discussion

This work presents comprehensive methods to detect yet unknown CLR/bacteria interactions that further enable to investigate the importance of CLRs in the recognition of and the response to bacterial pathogens, as shown here for the interaction of LTA-derived glycolipids from GAS and the CLR Mincle.

Sensing pathogens by CLRs expressed on APCs influences the immune responses in several regards, including antigen presentation, phagocytosis, complement activation and immune homeostasis (101). Therefore, identification of CLRs and their respective ligands that enhance or modulate immune responses by inhibition or activation of signaling pathways is of great interest. Bacteria express a huge repertoire of several carbohydrate-containing structures on their surface that are essential for bacterial growth and survival (182). Since CLRs are known to sense carbohydrate structures, bacteria present promising organisms to study the impact of CLRs in pathogen recognition and induction of signaling pathways. As an important initial step, the first project (chapter 3, paper II (183)) presents different in vitro methods that allow for identification of novel CLR/bacteria interactions. It is worth to mention that all methods can be easily applied to Gram-negative and Gram-positive bacteria, but each method clearly has its own advantages and disadvantages. The ELISA-based method relies on the immobilization of the bacteria on a plate which might lead to false-positive CLR-candidates due to protein aggregation. Although the ELISA offers the possibility for high-throughput screening, a following flow cytometry-based assay is recommended to confirm the respective CLR candidates. Application of an adequate gating strategy enables the identification of bacteria, hence ruling out debris or other contaminants in the sample. Nevertheless, only bacteria that are detectable by size in the flow cytometer can be analysed. The detection of small bacteria like mycoplasma may cause problems (184). Finally, a confocal microscopy-based method allows for co-localisation studies that directly visualize CLR/bacteria interactions (183).

All methods described are based on soluble fusion proteins, that consist of a Fc portion of a human IgG1 and the CRD of a CLR (178, 185). In clinical applications, fusion proteins are frequently used, since they offer several advantages. Favorably, the structure of the Fc part enables bivalent presentation of the ligand, hence increasing receptor avidity. Additionally,
the Fc part impacts important aspects of the fusion proteins like stability and solubility and folds autonomously (186). Furthermore, purification of the proteins is performed fast, simply and with low costs via the Fc region using well established protein-G/A affinity chromatography methods (187). Since the first discovery of a CD4-Fc fusion protein that prevented infection of T cells with human immune deficiency virus in 1989 (188), the interest in Fc-fusion molecules has been constantly increasing. Up to now, several Fc-fusions are already used in therapeutic applications (186). Here, fusion proteins are predominantly used to target specific receptors and mediate agonistic or antagonistic functions to enhance or inhibit immune responses, respectively. Among the approved Fc-fusion protein therapeutics, etanercept is applied as TNF-α-inhibitor that prevents proinflammatory responses in patients with severe, active rheumatoid arthritis (189). Furthermore, dulaglutide, a recombinant fusion protein consisting of a glucagon-like peptide-1 analog linked to a modified IgG4 Fc-fragment was recently approved as therapeutics to control the blood sugar level in patients with type 2 diabetes (190). A current approach is the fusion of peptides that specifically bind cancer cells to the Fc-domain to induce antibody-dependent cellular cytotoxicity (191).

Apart from their relevance in clinical applications, the use of Fc-fusion proteins in biomedical research has attracted increasing interest over the last decades. Since the interactions of CLRs with their carbohydrate ligands are characteristically weak, the expression as Fc-fusion proteins that form homodimers with bivalent CRD presentation increases the avidity of each protein (192). This enables even the detection of low affinity receptor/ligand interactions and renders Fc-fusion proteins interesting tools to analyse these interactions. For example, fusion proteins are used in microarrays to detect novel receptor ligands. These protein, lectin and glycan arrays rely on plate-coated biomolecules, like proteins as lectins, or carbohydrates. The binding of other proteins, cells, carbohydrates, antibodies or Fc-fusion proteins to these components is commonly evaluated by laser-based methods (193, 194). Recently, carbohydrate arrays including synthetically produced glycan structures as potential ligands and CLR-hFc fusion proteins were applied to evaluate the receptor binding preference in regard to ligand structure and multivalency (195, 196).

Applying a set of recombinant fusion proteins, several CLRs were shown to interact with bacteria like *S. pneumoniae* (158) or fungi such as *Malassezia pachydermatis* (197), *Gnadoderma lucidum* (198) and *Cordyceps sinensis* (198). Although several interactions of
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CLR receptors (CLRs) with pathogens are described, the specific ligand involved is still unknown in most cases. Since bacteria that express glycoconjugates in their surface structures harbor potential ligands for CLRs, these bacteria are of special interest in the field of CLR research. For instance, *C. jejuni* incorporates several different carbohydrates into important structures like the lipooligosaccharides (LOS), N and O-linked glycoproteins as well as the peptidoglycan and the polysaccharide capsule (199). One publication showed that N-glycosylated proteins and LOS containing a terminal N-acetylgalactosamine (GalNAc) derived from *C. jejuni* were recognized by the human (h) CLR macrophage galactose-type C-type lectin (MGL) (200). However, in the study presented in chapter 3, only minor binding of the murine (m)MGL-1 to the tested *C. jejuni* isolates was detected. One possible explanation for this might be the different binding profiles of MGL isoforms that are not only described for mMGL-2, that is more similar to hMGL, and mMGL-1 (201, 202) but also for other CLRs as the human DC-SIGN and its eight murine orthologs DC-SIGN-related proteins (SIGNRs) (139, 140). Despite SIGNR6, a pseudogene, and SIGNR4 all other SIGNR receptors are described to generally recognize glycan ligands similar to DC-SIGN (139). A similar observation was made for the human- and mouse-derived Dectin-1. Low-valency β-glucan parts of fucan and laminarin were able to activate hDectin-1 expressing cells, but not cells expressing the mDectin-1. Furthermore, stimulation studies with curdlan, a high-valency β-glucan, showed binding to both human and murine Dectin-1. Further assays including reciprocal mutagenesis analysis and substitution assays revealed that it is not the ligand-binding site of hDectin-1, but the intracellular domain of this receptor that mediates its sensitivity to low-valency β-glucan ligands. Interestingly, the substitution with the hDectin-1-specific amino acids in the intracellular domain rendered mDectin-1 sensitive to low-valency β-glucans (203).

Dectin-1 is a type II transmembrane protein that is mainly expressed by myeloid cells from the macrophage/monocyte and neutrophil lineages, but also to a lower extent by DCs and T cell subpopulations (167, 204). Activation of Dectin-1 includes several cellular functions like pathogen sensing, uptake and clearance. Since the cytoplasmic tail of Dectin-1 contains a hemITAM-motif (205), engagement of Dectin-1 leads to the activation of effector functions including the production of chemokines and cytokines, thus shaping immune responses (105, 174, 206). Applying the methods described in chapter 3 paper II and the CLR-hFc fusion protein library, Dectin-1 was identified as a receptor for *C. jejuni* (183). Interestingly, Dectin-1 is classically described as a major receptor that recognizes specifically β-1,3-
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glucans present in fungi like *C. albicans* or *S. cerevisiae* (53). Zymosan, a cell wall polymer of yeast like *S. cerevisiae*, is recognized by TLR2 and Dectin-1. Whereas Dectin-1 induces phagocytosis and ROS production that is enhanced by TLR2 signaling, TLR2 triggers inflammatory cytokine production via NFκ-B that is further augmented by Dectin-1 in return (207). Moreover, also a cooperation of TLR2 and Dectin-1 was described in the context of mycobacterial species (133, 208). Interestingly, a study with several virulent, attenuated and avirulent mycobacterial species showed that, in contrast to an anti-fungal immune response, Dectin-1 was not able to induce phagocytosis in macrophages upon mycobacterial sensing. However, the release of immune mediators by macrophages such as the cytokines IL-6, TNFα, RANTES and G-CSF was impaired in a Dectin-1-dependent manner (208). Strikingly, up to know there is no data suggesting that mycobacterial cell wall incorporates β-glucan. Furthermore, Dectin-1 is also known to interact with other bacteria like *Haemophilus influenzae* (209) or parasites, such as *L. infantum* and *L. amazonensis* (210, 211). These reports clearly demonstrate that Dectin-1 plays a role in recognition of other pathogens than fungi, but future work has to be performed to gain more insight into the relevance of Dectin-1 regarding recognition of bacteria, signal transduction and immune modulation. A huge progress will be the identification of distinct Dectin-1 ligand(s) in these pathogens, which facilitates research on how Dectin-1 affects immune responses towards pathogens beyond the focus on fungi (212).

Besides the use of Fc-fusion proteins, also reporter cell line assays are commonly applied to screen CLR/PAMP interactions, for their ligands and to validate CLR agonists or antagonists (132, 155, 157, 164, 213-216). These reporter cells express distinct CLRs on their surface coupled to an intracellular promotor-reporter expression cassette, e.g. NFAT-GFP. Here, ligand binding to the CRD of the CLR induces the activation of the transcription factor NFAT. Since subsequent activation of the GFP cassette leads to a fluorescence signal, CLR activation can be determined by measuring the GFP intensity (155, 164, 216). The application of reporter cell lines was for example used to extend the finding that *S. pneumoniae* is recognized by Mincle (158) by the discovery of *S. pneumoniae*-derived glucosyldiacylglycerol present in the LTA as Mincle ligand (157). Along that line, the second project (chapter 4, paper III (164)) contributed to the finding, that two LTA-derived components of GAS, namely DGDG and MGDG that differ in one glucose residue are recognized by Mincle. Moreover, the data suggest that the increased susceptibility of GAS
infected Mincle-deficient mice is probably due to an impaired ability to release inflammatory mediators (164).

GAS is an important human pathogen that expresses several adhesion molecules, like LTA, to mediate binding to host cells. In contrast, multiple host cells are known to play an important role in anti-GAS immunity by the release of various inflammatory cytokines in response to GAS infection to control bacterial growth and dissemination (217-219). Additionally, the production of NO and ROS increases the clearance of GAS in early stages of infection (220). Nonetheless, bacteria evolved during evolution efficient and complex evasion mechanisms to escape the host immune response, such as the expression of nucleases to prevent entrapment by neutrophil extracellular traps or the blockage of antimicrobial peptides (221, 222). Modification or masking of critical virulence factors is a common feature of pathogenic organisms to evade the immune system. For instance, it is assumed that GAS covers TLR2 agonists like LTA and peptidoglycan by the expression of a thick capsule to ensure immune evasion (223) until phagocytically active immune cells destroy the capsule (224). Also the fact that Dectin-1 was not able to detect some pathogenic yeasts like A. fumigatus or C. neoformans although they express β-glucans (225), argues for the hypothesis that these strains are able to mask their β-glucan to avoid recognition by PRRs (226). Moreover, phase variation is a common strategy of several C. jejuni strains to rapidly adapt to environmental changes and avoid recognition by host cells by controlling the expression of glycosylated LOS (227), capsule (228, 229) and several other bacterial determinants (230). Strain-specific genetic differences as well as mechanisms like phase-variation and antigenic variation that even frequently occur in each individual C. jejuni strain may additionally explain different study outcomes (183, 200, 231). Recognition of LTA-derived MGDG by Mincle initiated a protective immune response against GAS whereas sensing of DGDG suppressed this response without further initiation of signal transduction. Since it is hypothesize that DGDG serves as suppressor for agonistic PRR-mediated signaling via Mincle, it is tempting to speculate that bacterial strains might vary their ratio of MGDG and DGDG in the LTA to escape Mincle-mediated anti-bacterial immunity (164). Whereas it is shown for group B Streptococcus (GBS) that blockage of the glucosyltransferase, the GBS-DGDG synthesizing enzyme, led to attenuated virulence of the bacteria in vivo (232), there is no data existing concerning the precise function of GAS-DGDG.
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The paper III presented in chapter 4 reported that invasive and noninvasive GAS strains incorporate different DGDG/MGDG ratios in the LTA (164). Interestingly, amongst the tested GAS strains, those with increased amounts of DGDG belong to the group that caused invasive infections (5448, NIH34, NIH44), whereas strains with lower pathogenicity (SF370, K33, Se235) harbored comparably less DGDG. In correlation with this finding, it was shown that infection of mice with the different GAS strains influenced the clinical disease course, which might be partially explained by differences in the DGDG/MGDG ratio (164). Interestingly, infection of mice with the highly invasive strain 5448 (233, 234) that displayed the most striking difference in DGDG/MGDG ratio neither significantly initiated Mincle-dependent immune responses (164) nor impaired the disease progression of the infected mice (unpublished data), although this GAS strain was shown to be recognized by Mincle. Indeed, differences between in vitro and in vivo data are also well described in the context of other CLRs. For instance, infection of mice with S. pneumoniae in a model of invasive pneumococcal disease did not indicate a role for Mincle in anti-pneumococcal immune response in vivo although binding of Mincle in a Ca2+-dependent manner to S. pneumoniae was detected (158). The reasons for the discrepancy between in vitro and in vivo data are manifold. One explanation might involve the accessibility of the ligand since some ligands like MGDG and DGDG are incorporated into the LTA present in the bacterial membrane that is covered by the thick peptidoglycan (235) but can also be shed or exposed to the outer space of several streptococci (232, 236). Since Mincle did not recognise the whole LTA structure, it is likely that only portions of shed and degraded moieties are recognized. Most convincingly, the involvement of other PRRs than CLRs in signaling as well as the observation that other CLRs can compensate for missing signaling pathways is well known in literature. Thus, we cannot exclude the contribution of other PRRs in GAS recognition and subsequent initiation of signal transduction. For example, the CLR MCL is described to be co-expressed and -regulated with Mincle (237), both being upregulated upon MyD88 activation (238). Furthermore, whole LTA and the peptidoglycan are well known ligands for TLR2 in Gram-positive bacteria (239) and TLR2 has been shown to upregulate Mincle expression to induce downstream signaling upon stimulation with Corynebacterial-derived glycans (161). A clear role for the TLR adaptor protein MyD88 in initiating immune response towards GAS is described, since the absence of MyD88 caused a significantly decreased production of TNFα, type I IFNs, IL6 and IL12 by phagocytic immune cells. This impacted neutrophil recruitment and led to a higher susceptibility of MyD88-deficient mice challenged with GAS (240-243).
Additionally, impaired MyD88 expression in human patients affected TLR-mediated ligand recognition and may result in severe life-threatening predisposition to several pyogenic bacterial infections caused by e.g. *Staphylococcus aureus* and *S. pneumoniae* (244, 245). In contrast, whereas TLR9 was shown to play a protective role against GAS *in vivo* (181) the impact of TLR2 and TLR13 in anti-GAS immunity was not confirmed in a murine infection study (180, 217). Moreover, the different pathogenicity profiles of bacterial strains may explain the partially contradictory results. However, also technical features and the experimental design like the infection model, infection route and dose might influence the outcome of *in vivo* studies in general (246). Indeed, applying a model of focal pneumonia in infection of mice with a non-invasive *S. pneumoniae* strain revealed a protective role for Mincle as indicated by increased bacterial recovery in lungs and pro- and antiinflammatory cytokine production and larger areas of inflamed tissues in Mincle-deficient mice (157).

Since CLR activation impacts both, the phagocytic activity of APCs and the initiated immune responses, the knowledge about CLR/pathogen interactions and involved ligands was used in the last decades to design CLR agonists that specifically target CLR-expressing cells (247). Indeed, activation of CLRs has been shown to efficiently deliver drugs and genes into specific cells to initiate immune responses towards vaccines or tumors and also to induce immune tolerance (248). For example, antibody-mediated targeting of the plasmacytoid DC (pDC)-specific CLR blood DC antigen 2 (BDCA-2) on BDCA-2-humanized mice impaired CD4<sup>+</sup> and Treg responses (249). Major research on glycan-based CLR targeting has been performed for the CLR DC-SIGN. Due to its tetrameric CRD presentation, DC-SIGN allows multivalent targeting (101). Modification of antigens, nanoparticles, liposomes or dendrimers with DC-SIGN specific glycan ligands has already been successfully introduced to target DC-SIGN and influence T cell responses (250-256). Furthermore, recognition of glycan-harboring components as LPS, zymosan or TDB by PRRs is described to initiate expression of cytokines, enhance internalization of antigens and their presentation on MHC molecules to T cells, hence influencing the T cell fate (257). This modulatory function renders carbohydrate ligands interesting candidates for vaccine adjuvants and paves the way for the field of glycan-based vaccines. Vaccination of mice with liposomes harboring TDB as Mincle ligand mediated strong anti-GAS immunity after intranasal infection which was caused by a prominent Th17 immune response (258). GAS infection *in vivo* characteristically leads to a Th17 response that plays an important role in the protection against GAS (259,
260). Since MGDG was shown to induce a Th17 response (164), this novel Mincle ligand harbors potential as a future adjuvant candidate. It is known that Mincle recognizes mycobacterial-derived TDM as well as its synthetic analog TDB and plays an essential role in enhancing protective immune response via FcRγ/Syk/CARD9 axis in immune cells, emphasizing its potent adjuvant activity (131, 261). Studies using vaccines with TDB/TDM as adjuvants showed that both glycolipids mediate immunostimulatory effects towards *M. tuberculosis* that involves the FcRγ/Syk/CARD9 pathway and the activation of CD4+ T cells (262, 263). Up to now, TDB and TDM are applied in liposome-based adjuvants in experimental vaccines against pathogens like *M. tuberculosis*, *Chlamydia* and blood stage malaria (263-266).

To further investigate the nature of different Mincle ligands, studies were performed to analyse the crystal structures of human and bovine Mincle. Results showed that Mincle harbors a CRD that contains a central Ca2+ ion to complex the 3- and 4-OH groups of the glucose in the trehalose head-group. On the other site of this primary binding site, a secondary binding site complexes the second glucose residue in the trehalose head-group. Opposite to this binding pocket, a major hydrophobic groove binds to linear or branched trehalose glycolipids (143-146). Further studies analysed the influence of the ligand structure in regard to Mincle binding and signal transduction and showed that branched lipid backbones (267) with long acyl chains (144) are better complexed by Mincle and induced cytokine release by macrophages more efficiently (268, 269). Recently, different long-chain, α-branched trehalose mono- and diesters were synthesized to unravel their capability to be recognized by Mincle and to induce cell signaling. Interestingly, the prepared diesters of the trehalose glycolipid mediated an increased agonistic activity of hMincle compared to the adjuvant TDB (270). The knowledge from these structural data can be used in the future to develop novel Mincle agonists that mediate efficient adjuvant properties.

In general, CLR are described to be promiscuous receptors that are often able to detect more than one ligand (147). For example, Mincle recognizes various nonself-derived glycolipids and glycoproteins but also self-ligands such as sterols or β-glucosylceramides (213, 271, 272). Obviously, Mincle is also able to bind to the same ligand that derived from different bacterial species, as shown for the glucosyldiacylglycerol of GAS (164) and *S. pneumoniae* (157). Since glucosyldiacylglycerol is a part of the LTA which is a common feature of Gram-
positive bacteria, further studies will elucidate if this glycolipid is generally recognized by Mincle in all Gram-positive bacteria. However, there is insufficient knowledge about differences in ligand preferences within pathogen species as shown for the recognition of β-glucan harboring fungi by Dectin-1 (225). The mode of the initiated signal transduction by CLR engagement is dependent on several factors like receptor density and the nature of the respective ligands. For example, the affinity and avidity of ligand binding impairs signal quantity and duration mediated by CLRs. But also the ligand properties like soluble or particulate as well as the ligand size influence the resulting immune response (273). It was shown for Mincle that the expression of this receptor in unstimulated cells is only weak, whereas Mincle is upregulated upon stimulation and translocated to the cell surface via MyD88 (238). Previous studies have shown that the constitutively expressed CLR MCL enhances the expression of Mincle upon stimulation with TDM (274). In particular, upon activation, MCL and Mincle form a complex heterodimer through the binding of the hydrophobic repeat of MCL to the stalk region of Mincle, hence increasing the cell surface expression of Mincle (237, 238, 275). These data and the finding that MCL and Mincle both sense TDM (132, 274, 276) pinpoint to a synergistic relationship between these two receptors. Furthermore, it is hypothesized that Mincle upon a certain threshold fine-tunes the signaling that is initiated by MCL ligands to regulate host immune response (277).

In conclusion, the presented work highlights the role of CLRs in anti-bacterial immunity. Methods were described to detect CLRs that recognize bacteria. This further allows for functional characterization of CLR/bacteria interactions to unravel host/pathogen interactions, bacterial pathogenesis and host immune responses. The identification of a specific CLR ligand, as shown for GAS-derived MGDG and DGDG-binding to Mincle, as part of a collaborative effort enables additional approaches like glycan-based targeting and the production or the modification of potent vaccines against GAS.
Chapter 6: Future outlook

Although the previous chapters present examples that CLRs like Dectin-1 and Mincle mediate an important role in anti-bacterial immunity, still intense work needs to be done to identify the Dectin-1 ligand in *C. jejuni*, as well as the underlaying immune mechanisms and signaling consequences of the MGDG and DGDG/Mincle interaction in GAS.

Bacteria like *C. jejuni* constantly change their surface structures to adapt to new environments, including evasion of host immune responses. In addition to inter-strain specific modifications, also changes of surface structures within one specific strain are described. Studies are needed that reproducibly screen a broader set of *C. jejuni* strains that differ in various characteristics like genetics or lifestyle as generalists or specialists (183). Since this work only focused on the binding of Dectin-1 to *C. jejuni*, further studies are needed to show if this recognition also induces cell signaling or if it is dispensable for the initiation of an immune response as seen for *C. neoformans* (173) and other examples of CLR/pathogen interactions (136, 158, 176). Moreover, the distinct ligand for Dectin-1 present in *C. jejuni* has to be determined.

Regarding the detection of LTA-derived MGDG and DGDG as Mincle ligands as part of a scientific collaboration, it would be helpful if also a larger number of GAS strains isolated from clinical patients in various stages of infection are analysed concerning their MGDG/DGDG ratio to enable association between virulence and Mincle-ligand expression. Since LTA is a ubiquitous component of the Gram-positive cell wall (278), it is interesting to investigate if Mincle also mediates recognition of other Gram-positive bacteria via LTA-derived MGDG. The strain 5448 was the only GAS strain investigated with a minor role for Mincle in vivo despite it was recognized by this receptor (164). The GAS strain 5448 was isolated from a patient with severe, invasive necrotizing fasciitis (234). Since the infection and inflammation of the skin is known to cause lower oxygen content as under normal condition, so-called hypoxia, one should consider the oxygen concentration to obtain physiologically relevant results. Indeed, publications report that hypoxia-inducible factor-1 (HIF-1), the main transcription factor that regulates the adaptive immune response to hypoxia has an impact on both, the susceptibility to GAS and the PRR signaling pathways. On the one hand, GAS 5448 induces HIF-1α (279). A murine infection study showed that HIF-1-
deficient mice are more susceptible to an invasive GAS 5448 infection, probably due to a decreased phagocytic killing of the bacteria (279). On the other hand, crosstalk of HIF with PRRs like TLR2 and TLR6 (280) is described in the literature as well as the HIF-induced upregulation of TLR4 expression in macrophages (281). Interestingly, in response to activation via TDB, Mincle signaling increased the transcription of the hif1α gene but was not required for the expression of HIF-1α (282). Furthermore, key regulator molecules like the adaptor molecule CARD9 and the transcription factor NFκ-B that play an important role in signaling via Mincle and other CLRs were found to be involved in hypoxia-sensitive pathways (283-285). Further studies should unravel the role of Mincle in GAS infection under hypoxic conditions.
Concluding remarks

Chapter 7: Concluding remarks

The presented projects highlight the relevance of CLRs in sensing bacterial pathogens like C. jejuni or GAS and contributed to unravelling that GAS-derived MGDG and DGDG serve as novel ligands for Mincle.

CLR-Fc fusion proteins present powerful tools to identify yet unknown CLR/bacteria interactions. Parts of this thesis describe ELISA-, flow cytometry- and confocal microscopy-based methods that enable high-throughput screening and subsequent confirmation of CLR candidates as innate bacterial sensors based on a set of human and murine CLR-Fc fusion proteins. With the help of these methods, Dectin-1 was detected as a CLR that recognises different C. jejuni isolates.

Additionally, this thesis contributed to the finding that the LTA-derived glycolipids MGDG and DGDG from GAS act as novel ligands for Mincle. Whereas MGDG induced activation of innate immune responses against GAS, DGDG interfered negatively with this response. Moreover, in vivo data showed that Mincle mediated a protective role in anti-GAS immunity.

The identification of distinct bacterial CLR ligands is important for future vaccination strategies to induce potent immune responses. Moreover, the analysis of initiated immune responses by receptor/ligand interactions offers the possibility to therapeutically interfere with the activated signaling pathway and thus shape immune responses.
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Affidavit

I herewith declare that I autonomously carried out the PhD-thesis entitled “Role of C-type lectin receptors in bacterial recognition”.

No third party assistance has been used.

I did not receive any assistance in return for payment by consulting agencies or any other person. No one received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis.

I conducted the project at the following institution: Immunology Unit & Research Center for Emerging Infections and Zoonoses, University of Veterinary Medicine Hannover, Foundation.

The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.

I hereby affirm the above statements to be complete and true to the best of my knowledge.
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As we express our gratitude, we must never forget that highest appreciation is not to utter words, but to live by them. -John F. Kennedy-

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