Sensing of Bacterial Peptidoglycan by Peptidoglycan Recognition Molecules

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

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2013

Abstract

Peptidoglycan is an essential component of bacteria that provide structure and integrity of the cell. The main goal of this thesis has been to study the innate immune molecules that recognize bacterial peptidoglycan. In particular, the first three data chapters focus mainly on Nod1 and Nod2, the intracellular innate immune receptors critical for peptidoglycan recognition. First, the contribution of Rip2, the adaptor protein downstream of both Nod1 and Nod2 signaling, in innate and adaptive immunity was investigated in vivo using Rip2-deficient mice. I demonstrated that Rip2 was required and critical for triggering Nod1- and Nod2-driven Th2 immunity in vivo. Second, the mechanism by which Nod1 and Nod2 ligands enter into epithelial cells was investigated. Using biochemical approaches and molecular biology techniques in vitro, I determined that the entry of these Nod1 and Nod2 ligands was mediated through clathrin-dependent endocytosis, and the internalization of the ligands was dependent on pH. More importantly, I identified a role for SLC15A4, an oligopeptide transporter expressed in early endosomes, in Nod1-dependent signaling. The last chapter of this thesis focused on delineating the role of the poorly characterized peptidoglycan binding and amidase protein PGLYRP2 in innate immune responses to Salmonella enterica serovar Typhimurium infection. Although PGLYRP2 did not seem to modulate Nod1- and Nod2-dependent early Th17 inflammatory
responses during *Salmonella* colitis in vivo, the protein was shown to have a protective role in the host defense against *S. Typhimurium*, and this role appeared to be independent of its amidase activity. All together, the work presented in this thesis provides novel insights into various aspects of signaling mechanisms dependent on Nod1, Nod2, as well as the poorly characterized mammalian PGLYRP2 in mucosal innate immunity.
Acknowledgments

I would like to dedicate this thesis to my parents, IL-Beom Lee and Young-Hee Lim, for their unconditional love and support throughout the years.

Completing a PhD degree is no small feat. In my case, it was a particularly challenging journey, compounded by the fact that I had to switch labs two years into my program. But there were individuals whom I met along the way that believed in me, and gave me strength to continue my study; and it is because of them, I am here today with a PhD in my pocket.

First and foremost, I am forever indebted to my amazing supervisor, Dr. Stephen Girardin, for having the vision and taking me on as his graduate student. Five years ago, Steph saw something in me that even I was not sure of, and took a big chance on me when it was probably not in his favor to do so. Combined with his passion for science, Steph’s incredible mentorship provided the perfect environment for me to grow tremendously not only as a scientist, but also as a better person. When it comes down to it, I am where I am today because of Steph. Thank you for believing in me all these years, Steph!

I would like to thank Dr. Dana Philpott who has also been instrumental to my success in obtaining my degree. Her kind heart and compassion were what got me through during difficult times, and I feel incredibly lucky to have had her as a mentor and a friend.

Many thanks to my committee members Dr. Ian Crandall and Dr. Jeremy Mogridge for their guidance and constructive feedback of my research. The department of LMP has also been wonderful to me throughout my graduate study. Especially, I am very greatful to Dr. Harry Elsholtz, and Rama Ponda for their support.

I could not have survived graduate school without the support from past and present members of both the Girardin and the Philpott lab. I am very fortunate to have met and shared a large part of my degree with amazing group of people, especially Kavi Ramjeet, Sue Robertson, Fraser Soares, Kaoru Geddes and Matt Sorbara. Thanks for all those long hours of interesting conversations, whether it be about science or life in general, and thus, making my graduate school life all the more exciting and enjoyable.
Ultimate Frisbee has played an integral part during my PhD. This sport provided a sanctuary for me, a place where I could go and completely unwind myself. Through playing the sport, I also met so many wonderful people, and some of the friendships I formed, I hope to continue for life. I am grateful to have been a part of Tickle Me Stalin for a chance to meet fantastic group of people, including Wes & Meech, and Melatron. Moreover, I am so thankful for my wonderful group of four from Torontula, Nancy So, Kate Jardine, Michelle Po, and Melissa MacPherson; you are not only amazing scientists, but also incredible athletes who believe that hard work always pays off in the end. You showed me how to stand tall as a woman in the field of science.

Last but not least, I cannot thank my parents enough. Without their support and encouragement for so many years, I would not have a PhD today. I have the utmost respect for them, for having the guts to leave behind a 16-year-old girl all by herself in Canada, because they believed she could achieve anything she set her mind to it. I love you, mom and dad.
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Dissemination of Work Arising from this Thesis

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Chapter 4 was published as:


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Chapter 6 was published as:


Additional publications arising during the course of graduate studies:

Rubino, S., Lee J., Girardin S.E. Mammalian PGRPs also mind the fort. *Cell Host & Microbe* 2010 8(2): 130-2.
### List of Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>AGS</td>
<td>human gastric epithelial cells</td>
</tr>
<tr>
<td>ALRs</td>
<td>absent-in-melanoma 2 (AIM2)-like receptors</td>
</tr>
<tr>
<td>APAF1</td>
<td>apoptotic protease activating factor 1</td>
</tr>
<tr>
<td>APCs</td>
<td>antigen presenting cells</td>
</tr>
<tr>
<td>BIRs</td>
<td>baculovirus inhibitor repeats</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase recruitment domain</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>cIAP1/cIAP2</td>
<td>cellular inhibitor of apoptosis 1 and 2</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptors</td>
</tr>
<tr>
<td>CYLD</td>
<td>Cylindromatosis</td>
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<tr>
<td>DAMPs</td>
<td>danger-associated molecular patterns</td>
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<tr>
<td>DAP</td>
<td>diaminopimelic acid</td>
</tr>
<tr>
<td>DGGE</td>
<td>denaturing gradient gel electrophoresis</td>
</tr>
<tr>
<td>DKO</td>
<td>double knockout</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DSS</td>
<td>dextran sulfate sodium</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
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<td>ELISPOT</td>
<td>Enzyme-linked immunosorbent spot</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular receptor kinase</td>
</tr>
<tr>
<td>FAE</td>
<td>follicle-associated epithelium</td>
</tr>
<tr>
<td>FCA</td>
<td>Freund’s Complete Adjuvant</td>
</tr>
<tr>
<td>FK156</td>
<td>d-lactyl-L-Ala-g-D-Glu-meso-DAP-Gly</td>
</tr>
<tr>
<td>GALT</td>
<td>gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglycosamine</td>
</tr>
<tr>
<td>GWAS</td>
<td>genome-wide association studies</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>IECs</td>
<td>intestinal epithelial cells</td>
</tr>
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<td>IELs</td>
<td>intraepithelial leukocytes</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IKK</td>
<td>inhibitor of KappaB Kinase</td>
</tr>
<tr>
<td>IMD</td>
<td>immune deficiency</td>
</tr>
<tr>
<td>ITCH</td>
<td>atrophin-1 interacting protein 4, AIP4</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal Kinase</td>
</tr>
<tr>
<td>KC</td>
<td>keratinocyte-derived chemokine</td>
</tr>
<tr>
<td>Lcn2</td>
<td>lipocalcin 2</td>
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<tr>
<td>LP</td>
<td>lamina propria</td>
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<td>LPLs</td>
<td>lamina propria lymphocytes</td>
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<td>LPS</td>
<td>lipopolysaccharides</td>
</tr>
<tr>
<td>LRRs</td>
<td>C-terminal leucine-rich repeats</td>
</tr>
<tr>
<td>LTA</td>
<td>lipoteichoic acids</td>
</tr>
<tr>
<td>LUBAC</td>
<td>the linear ubiquitin chain assembly complex</td>
</tr>
<tr>
<td>MAMPs</td>
<td>microbial-associated molecular patterns</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen-activated protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>M cell</td>
<td>microfold cell</td>
</tr>
<tr>
<td>MDP</td>
<td>muramyl dipeptide</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MurNAc</td>
<td>N-acetylmuramic acid</td>
</tr>
<tr>
<td>NACHT</td>
<td>domain present in neuronal apoptosis inhibitor protein (NAIP), the major histocompatibility complex (MHC) class II transactivator (CIITA), HET-E and TP1</td>
</tr>
<tr>
<td>NBD</td>
<td>nucleotide-binding domain</td>
</tr>
<tr>
<td>NEMO</td>
<td>NFκB essential modulator</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
</tr>
<tr>
<td>NLRs</td>
<td>Nod-like receptors</td>
</tr>
<tr>
<td>Nod</td>
<td>Nucleotide-binding oligomeriazation domain</td>
</tr>
<tr>
<td>OMVs</td>
<td>outer membrane vesicles</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PMNs</td>
<td>polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>PAI</td>
<td>cag-pathogenicity island</td>
</tr>
<tr>
<td>PGRPs</td>
<td>peptidoglycan recognition proteins</td>
</tr>
<tr>
<td>PPs</td>
<td>Peyer’s Patches</td>
</tr>
<tr>
<td>PRMs</td>
<td>pattern recognition molecules</td>
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<td>PRRs</td>
<td>pattern recognition receptors</td>
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<td>PYD</td>
<td>pyrin domain</td>
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<tr>
<td>qPCR</td>
<td>Quantitative real-time PCR</td>
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<td>RegIIγ</td>
<td>regenerating islet-derived IIγ</td>
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<td>receptor-interacting protein 2</td>
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<td>retinoic acid-inducible gene-I (RIG)-like receptors</td>
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<td>RNAi</td>
<td>RNA interference</td>
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<td>SLC</td>
<td>the solute carrier</td>
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<td>SPI-2</td>
<td>pathogenicity island 2</td>
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<td>transforming growth factor β-activated kinase</td>
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<td>TCT</td>
<td>GlcNAc-AnhydroMurNAc-TetraDAP</td>
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<td>TLRs</td>
<td>Toll-like receptors</td>
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<td>TNBS</td>
<td>trinitrobenzene sulfonic acid</td>
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<td>TRAF</td>
<td>tumor necrosis factor receptor-associated factor</td>
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<td>T regulatory cells</td>
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<tr>
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<td>terminal restriction fragment length polymorphism</td>
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<td>type III secretion systems</td>
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<td>ulcerative colitis</td>
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<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>XLP-2</td>
<td>X-linked lymphoproliferative syndrome type 2</td>
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Chapter 1
Introduction

1

1.1 Peptidoglycan

Peptidoglycan is an essential part of cell wall in almost all bacteria. Gram-positive bacteria have a thick cell wall composed of 20 to 80 layers of peptidoglycan, as well as teichoic acids, lipoteichoic acids, and lipoproteins (Figure 1.1). In Gram-negative bacteria, peptidoglycan is found as a thin layer, located in the periplasmic space between the plasma membrane and the outer membrane (Figure 1.1). Peptidoglycan not only preserves cell integrity and maintains a defined cell shape, but also provides a scaffolding structure for anchoring other components such as lipoproteins and teichoic acids (Vollmer et al., 2008). Interestingly, peptidoglycan is one of the major components of bacteria readily detected by host pattern recognition receptors (PRRs), along with other components such as lipopolysaccharides (LPS) of the outer membrane from Gram-negative bacteria, and lipoteichoic acids (LTA) from Gram-positive bacteria.

1.1.1 General structure of peptidoglycan

Peptidoglycan is composed of glycan chains that are made up of alternating, (β-1-4)-linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) sugar residues linked to a short peptide chain at the lactyl group of MurNAc. Subtle changes in the peptide chain composition can occur between species (Schleifer and Kandler, 1972), however, this peptide stem is typically composed of four to five amino acids starting with L-Ala and D-Glu as the first and the second amino acids, respectively. In contrast, the third residue of the stem peptide is a lysine in coccoid Gram-positive bacteria such as Staphylococcus and Streptococcus species, but a meso-diaminopimelic acid (DAP) residue in both Gram-negative and several rod-shaped Gram-positive bacteria such as Listeria and Bacillus species (Boneca, 2005; Girardin et al., 2003c). The two tetrapeptides \((\text{L-Ala-}\gamma\text{-D-Glu-meso-DAP (or L-Lys-)}\gamma\text{-D-Ala)})\) subunits are linked to each other through the branching diamino group containing residue to the \(\text{D-Ala}\) residue (Mengin-Lecreulx and Lemaitre, 2005) (Figure 1.2). Interestingly, the peptide stem mostly found in Gram-negative bacteria is a perfect example of a microbial-derived molecular signature, since it has features that are absent in eukaryotes: a \(\text{D}\)-form amino acid, a \(\gamma\)-type peptide bond, and meso-DAP. Moreover,
The Gram-negative cell wall is composed of a thin layer of peptidoglycan and an outer membrane embedded with molecules such as phospholipids, LPS, and lipoproteins. The Gram-positive cell wall is a dense layer typically composed of multiple layers of peptidoglycan, and molecules of lipoteichoic acid, teichoic acid and surface proteins. Adapted, with permission, from Dr. Kaiser’s microbiology website (http://student.ccbcmd.edu/~gkaiser/goshp.html).

Figure 1.1. The Gram-negative and Gram-positive bacterial cell wall.
A. A schematic structure of peptidoglycan. (B) The basic structure of peptidoglycan. The chemical structure of DAP-type peptidoglycan, common in Gram-negative bacteria is shown. The red inset shows the changes in structure common in Gram-positive bacteria, including the change of m-diaminopimelic acid to L-Lysine, and D-glutamic acid to D-iso-glutamine. (B) Adapted, with permission, from Immunological Reviews (Sorbara and Philpott, 2011).
the sugar group, MurNAc, is a hexose, which is only present in bacteria (Vollmer et al., 2008).

1.1.2 Adjuvant properties of muropeptides

Long before the discovery of peptidoglycan recognition molecules such as Nucleotide-binding oligomeriazation domain 1 (Nod1) and Nod2, smaller components of peptidoglycan containing MurNAc (also called muropeptides) were shown to be biologically active (Adam et al., 1974; Ellouz et al., 1974). DAP-containing tetrapeptides and muramyl dipeptide (MDP), which is composed of MurNAc and two amino acids, L-Ala and D-Glu (or D-isoGln), were initially discovered to act as adjuvants, an agent that enhances the immune-stimulatory response elicited by compounds having few effects on their own (Adam et al., 1974; Ellouz et al., 1974). Further, MDP was found to be the minimal peptidoglycan-derived structure required for eliciting adjuvant activity and could therefore replace Freund’s Complete Adjuvant (FCA), one of the most potent adjuvants and yet a highly toxic compound, for its ability to induce both humoral and cellular activity (Adam et al., 1981). Indeed, these muropeptides are effective adjuvants and thus, may be used for boosting the potency of drugs and vaccines. In particular, MDP and other muropeptides (tri- and tetrapeptides) enhance the expression of surface markers necessary for cell adhesion and antigen presentation, which leads to increased phagocytic and antimicrobial activity (Darcissac et al., 1996; Heinzelmann et al., 2000; O'Reilly and Zak, 1992). Furthermore, they elicit immune responses by increasing production of cytokines such as interferon (IFN)-γ, thereby stimulating lymphocyte activation (Saiki and Fidler, 1985; Souvannavong et al., 1990; Traub et al., 2006). Muropeptides have also been shown to display strong synergy with other ligands, such as lipopolysaccharide (LPS), thus eliciting a greater immune response in vitro in human primary cells (Vermeulen et al., 1987; Wang et al., 2001; Yang et al., 2001).

1.2 Nod1 and Nod2 as sensors of peptidoglycan

1.2.1 Nod-like receptors (NLRs)

Innate immune system is activated when danger- and microbial-associated molecular patterns (DAMPs and MAMPs) are detected by pattern recognition molecules (PRMs) on cells including epithelial cells, macrophages, and dendritic cells. Several families of secreted or cellular PRMs have been identified, and among these, cellular PRMs can be grouped into Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Nod-like receptors (NLRs), retinoic acid-inducible
gene-I (RIG)-like receptors (RLRs), and a new family termed absent-in-melanoma 2 (AIM2)-like receptors (ALRs) (Huysamen and Brown, 2009; Kumar et al., 2009; Schroder et al., 2009; Schroder and Tschopp, 2010; Wilkins and Gale, 2010). The membrane-bound TLRs, the first class of cellular PRMs to be identified, have been extensively studied in the last fifteen years; they are involved in the recognition of MAMPs such as LPS, flagellin, lipoproteins, DNA and RNA. On the other hand, the NLRs and the RLRs are intracellular cytosolic sensors, which are critical at monitoring DAMPs and MAMPs when insults evade extracellular surveillance.

1.2.2 Structure of Nod1 and Nod2

The Nod domain was first found in apoptotic protease activating factor 1 (APAF1) and its nematode homologue CED-4, which are regulators of developmental and p53-dependent programmed cell death (Derry et al., 2001; Inohara et al., 1999). Subsequently, two Nod containing molecules, Nod1 (Card4) and Nod2 (Card15), were identified through database searches for APAF1/CED4 homologues. The human NLR family consists of 22 proteins that can be subdivided into five subfamilies, NLRA, NLRB, NLRC, NLRX, and NLRP, according to the nature of their N-terminal effector domain (Figure 1.3) (Werts et al., 2011). NLRs are multi-domain proteins composed of a variable N-terminal effector region consisting of caspase recruitment domain (CARD), pyrin domain (PYD), acidic domain, or baculovirus inhibitor repeats (BIRs), a centrally located NACHT domain [domain present in neuronal apoptosis inhibitor protein (NAIP), the major histocompatibility complex (MHC) class II transactivator (CIITA), HET-E and TP1] that is critical for activation, and C-terminal leucine-rich repeats (LRRs) that senses MAMPs. Nod1 and Nod2 belong to NLRC family; Nod1 (NLRC1) has a single amino-terminus CARD domain, whereas Nod2 (NLRC2) has two amino-terminus CARD domains, followed by a central NACHT domain, and a C-terminal LRR domain (Figure 1.3).

1.2.3 Nod1 and Nod2 ligand specificity

Initially Nod1 and Nod2 were thought to recognize LPS intracellularly (Girardin et al., 2001; Inohara et al., 2001; Ogura et al., 2001), but it was soon discovered that LPS preparations were contaminated with other bacterial products. Subsequently, Nod1 and Nod2 were shown to detect specific motifs from bacterial peptidoglycan. Nod1 recognizes meso-DAP-containing peptidoglycan fragments (Chamaillard et al., 2003; Girardin et al., 2003a), to detect Gram-negative bacteria and certain Gram-positive bacteria, including Listeria and Bacillus species
Figure 1.3. The human NLR family.

Organization of the human NLR family according to structural similarities of their proteins. Adapted, with permission, from *Nature Immunology* (Kufer and Sansonetti, 2011).
(Girardin et al., 2003c). On the other hand, Nod2 recognizes MDP, which is the minimal structural component commonly found in peptidoglycan of both Gram-positive and Gram-negative bacteria (Girardin et al., 2003b; Inohara et al., 2003) (Figure 1.4).

Nod1 is ubiquitously expressed in many cell types including stromal and hematopoietic cells while Nod2 expression is believed to be mainly restricted to leukocytes. However, expression of Nod2 is induced by inflammatory signals in paneth cells (Ogura et al., 2003), and epithelial cells of the intestine (Hisamatsu et al., 2003), suggesting that Nod2 is important for mucosal homoeostasis.

1.2.4 Cellular localization of Nod1 and Nod2

Nod1 and Nod2 lack transmembrane domains and therefore, are considered cytoplasmic proteins. Although the exact location of these receptors in the cytoplasm is not clear, Nod1 (Kufer et al., 2008) and Nod2 (Barnich et al., 2005) have been shown to be associated with plasma membrane. Nod2 3020insC mutant, a truncated form of Nod2 associated with Crohn’s disease, was defective in sensing MDP (Girardin et al., 2003b; Inohara et al., 2003), and remained in the cytoplasm (Barnich et al., 2005). Further studies have linked membrane targeting of Nod2 and NF-κB activation (Lecine et al., 2007; Legrand-Poels et al., 2007), and also showed that localization of Nod2 to the plasma membrane promotes membrane recruitment of its adaptor protein, Rip2 (Lecine et al., 2007). Similar results were also reported for Nod1, as endogenous Nod1 was found in both the cytoplasm and the plasma membrane of AGS gut epithelial cells, at the site of bacterial entry (Kufer et al., 2008). Interestingly, it is likely that a correlation between host endomembrane targeting and innate immune function represents a general feature for not only Nod1 and Nod2, but also many NLR proteins (Philpott and Girardin, 2010).

1.2.5 Mechanism of the entry of Nod1 and Nod2 ligand

Entry of Nod1 and Nod2 ligands into the cytosol is essential for activation of Nod1 and Nod2. Supporting this, Nod1 in epithelial cells was shown to be activated by invasive Shigella flexneri, but not non-invasive strains (Girardin et al., 2001). In agreement, Nod1 and Nod2 have been shown to be critical at mounting immune responses against invasive bacteria, such as Shigella flexneri (Girardin et al., 2001), Listeria monocytogenes (Boneca et al., 2007; Opitz et al., 2006), Streptococcus pneumonia (Opitz et al., 2004), and Salmonella enterica serovar Typhimurium
Nod1 detects muramyl tripeptides in which the third amino acid is a \textit{meso} diaminopimelic acid (mesoDAP), a structure found in most Gram-negative bacteria. In contrast, Nod2 detects MDP, which is composed of a MurNAc sugar linked to the dipeptide \text{L-Ala-D-Glu}. While Nod2 can also detect muramyl tripeptides if the third amino acid is a lysine (found in Gram-positive bacteria), MDP is the minimal structure detected by Nod2. The difference between mesoDAP and lysine lies in a single carboxy group (highlighted in an ellipse). Contrary to Nod2, Nod1 detection does not require the presence of the sugar moiety. Also, even the first amino acid, \text{L-Ala}, is not absolutely critical. Thus, the minimal motif detected by Nod1 is the dipeptide \text{D-Glu-mesoDAP} (also known as iE-DAP).
(Geddes et al., 2010; Le Bourhis et al., 2009). In addition, Nod1 and Nod2 have been shown to detect non-invasive bacterial pathogens (Viala et al., 2004). In these cases, several mechanisms account for the delivery of extracellular peptidoglycan to the cytosolic compartment where Nod1 and Nod2 reside (Figure 1.5).

**SLC15 family of peptide transporters**

SLC family proteins, with over 300 transporters, transport short chain peptides and other organic small molecules from the extracellular space to the cytosol (Hediger et al., 2004). Based on the solute specificity, only a few out of the 46 SLC sub-families (from SLC1 to SLC46) could be potentially implicated in the transport of peptidoglycan-derived peptides. Among these are the 4 members of SLC15 family (H+-coupled oligopeptide transporter), the 20 members of SLC21 (organic anion transporting family) and the 18 members of SLC22 (organic anion/zwitterions/cation transporting family) proteins (Hediger et al., 2004). SLC15 family proteins, namely SLC15A1 (PepT1), SLC15A2 (PepT2), SLC15A3 (PHT2) and SLC15A4 (PHT1), deliver short-chain peptides by utilizing proton-motive force (Hediger et al., 2004). Human SLC15A1 and SLC15A2 have very broad substrate specificity, and are known to mediate the active translocation of di- or tri-peptides across epithelial tissues (Biegel et al., 2006; Meredith and Price, 2006). The poorly studied SLC15A3 and SLC15A4 have been shown to transport free histidine and certain oligopeptidates (Hediger et al., 2004). SLC15A1 is highly expressed in gut epithelial cells (Fei et al., 1994; Ismair et al., 2006), particularly in the proximal small intestine, whereas SLC15A2 is abundantly expressed throughout the epithelial cells in the lung and kidney (Biegel et al., 2006). Both SLC15A3 and SLC15A4 are widely expressed in various tissues, including tissues in the human gastrointestinal tract (Bhardwaj et al., 2006; Herrera-Ruiz et al., 2001). Interestingly, the expression of colonic SLC15A1 and SLC15A4 is upregulated in patients with ulcerative colitis and Crohn’s disease (CD) (Lee et al., 2009; Merlin et al., 2001). Studies have shown that SLC15A1 mediates the transport of Tri-DAP and MDP to activate Nod1-, and Nod2-dependent NF-κB signaling respectively, in human colonic Caco2/bbe epithelial cells (Dalmasso et al., 2010; Vavricka et al., 2004), although SCL15A1 does not transport Nod1 ligands in oocytes from *Xenopus laevis* (Ismair et al., 2006). Moreover, in human lung epithelial cells, SLC15A2 was shown to mediate the uptake of Nod1 ligand, iE-DAP (Swaan et al., 2008). In addition, we have recently showed that the oligopeptide transporter SLC15A4 expressed in the early endosome, was implicated in the transport of Nod1 ligands.
Figure 1.5. Entry mechanism for extracellular peptidoglycan.

Several mechanisms have been proposed for the entry into epithelial cells, including clathrin-mediated endocytosis, oligopeptide transporters (hPepT1 and SLC15A4), bacterial Type IV secretion systems, and outer membrane vesicles (OMVs). Refer to the text for detail. Adapted, with permission, from Immunological Reviews (Sorbara and Philpott, 2011).
Endocytosis

Endocytosis is a fundamental process that is used by eukaryotic cells to internalize molecules (Doherty and McMahon, 2009). Several entry mechanisms have been observed, including the most studied clathrin-mediated endocytosis, as well as caveolae-mediated endocytosis, phagocytosis and macropinocytosis (Doherty and McMahon, 2009). Two reports have demonstrated that Nod1 and Nod2 ligands were internalized via clathrin-mediated endocytosis in macrophages and epithelial cells (Lee et al., 2009; Marina-Garcia et al., 2009). In macrophages, Marina-Garcia et al. showed that MDP uptake occurred through clathrin-dependent endocytosis (Marina-Garcia et al., 2009), and trafficking of MDP required V-ATPases and endosomal acidification for Nod2 activation. In contrast, Lee et al. showed that the late-stage endosome maturation was not required in epithelial cells, since inhibiting the action of V-ATPases did not diminish Nod1 or Nod2 activation (Lee et al., 2009) (Chapter 4). Interestingly, a recent report demonstrated that intact polymeric peptidoglycan, rather than monomeric MDP or soluble peptidoglycan, can also be taken up into the innate immune cells (not nonphagocytic epithelial cells) by phagocytosis, and undergoes lysosomal degradation to be transported to the cytosol to activate Nods (Iyer and Coggeshall, 2011).

Bacterial secretion system

Another mechanism, used by Helicobacter pylori, to deliver peptidoglycan into the cell, is through bacterial secretion system. Helicobacter pylori is a Gram-negative bacteria that colonizes human gastric mucosa (Watanabe et al., 2010b). Infection with H. pylori is associated with gastric inflammation and ulceration, often resulting from the interplay between bacterial virulence factors and host immune responses. The cag-pathogenicity island (PAI) is one of the key virulence factors of H. pylori that encodes a type IV secretion system, through which effectors, such as CagA (Odenbreit et al., 2000) and peptidoglycan (Viala et al., 2004), are translocated into the host cell. Although H. pylori is an extracellular pathogen, epithelial
infection with the organism results in Nod1 activation (Allison et al., 2009; Viala et al., 2004). The mechanism by which *H. pylori* peptidoglycan enters epithelial cells is through type IV secretion system. Indeed, Viala et al. found that *H. pylori* expressing functional cag-PAI efficiently delivered radio-labeled peptidoglycan into the gastric epithelial cells, whereas *H. pylori* strain harboring nonfunctional cag-PAI failed to deliver radio-labeled peptidoglycan. The cag-dependent Nod1 activation plays an important role in host defense, since Nod1-deficient mice had higher bacterial loads during infection with cag-positive *H. pylori* strains. Moreover, Grubman et al. further supported these findings by showing that infection with *H. pylori* induced Interleukin (IL)-8 production by the gastric epithelial cell line, AGS cells, in a Nod1/cag-PAI-dependent manner (Grubman et al., 2009).

*Outer membrane vesicles (OMVs)*

Another mechanism, used predominantly by Gram-negative bacteria, to deliver peptidoglycan into the environment, is the release of outer membrane vesicles containing secreted products of the periplasm (Clarke et al., 2011). Recent studies provide evidence that peptidoglycan can also be delivered to epithelial cells by bacterial OMVs (Allison et al., 2009; Bielig et al., 2011; Kaparakis et al., 2010). OMVs are shed by Gram-negative bacteria during normal growth (Kuehn and Kesty, 2005), and contain numerous components of the bacterial cell wall including peptidoglycan (Zhou et al., 1998). Kaparakis et al. showed that OMVs from *Helicobacter pylori*, *Pseudomonas aeruginosa*, and *Neisseria gonorrhea* enter epithelial cells via lipid rafts to transport their peptidoglycan to Nod1 and to trigger Nod1-dependent NF-κB activation. Similarly, Bielig and colleagues demonstrated that *Vibrio cholera* OMVs are taken up by epithelial cells and induce Nod1-dependent inflammatory responses, although the mechanism by which OMVs enter the cell is not clear (Bielig et al., 2011).

1.2.6 Nod1 and Nod2 activation and signaling

In the absence of ligands, Nod1 and Nod2 are thought to be kept in an inactive state by intramolecular interactions (Hsu et al., 2008; Tanabe et al., 2004). Although the exact mechanism of peptidoglycan detection is unclear, direct binding of Nod1 and Nod2 to their ligand, Tri-DAP and MDP, respectively, has been recently demonstrated using purified recombinant proteins (Askari et al., 2012; Laroui et al., 2011; Mo et al., 2012). Specifically, Nod1 ligand, Tri-DAP, has been shown to interact with the C-terminal leucine-rich repeat (LRR) domain of Nod1.
(Askari et al., 2012; Laroui et al., 2011), which supports a previous study suggesting that Tri-DAP associates with the LRR of Nod1 (Girardin et al., 2005). Interestingly, the binding to MDP was recently shown to be dependent on the nucleotide-binding domain (NBD) of Nod2 (Mo et al., 2012), rather than the LRR domain, where the binding had been thought to take place previously (Tanabe et al., 2004). Upon ligand recognition, Nod1 and Nod2 undergo conformational changes (Inohara et al., 2005) leading to self-oligomerization mediated through the central NACHT domain. This is followed by the recruitment and activation of the serine threonine kinase RICK (Rip2), which associates with Nod1 or Nod2 through CARD-CARD interaction. The central pathway of Nod1 and Nod2 signaling involves this adaptor protein, Rip2, which is essential for initiating ubiquitin-dependent signaling events that activate pro-inflammatory pathways such as NF-κB and the mitogen-activated protein (MAP) kinases p38, JNK and ERK (Girardin et al., 2001; Inohara et al., 2000; Kobayashi et al., 2002).

Activation of Rip2 is controlled by ubiquitination, which requires three enzymatic steps performed by E1, E2, E3 enzymes (Chen, 2005). Upon ligand binding, the recruitment of the E3-ubiquitin ligases including TRAF2/TRAF5 (Hasegawa et al., 2008), cIAP1/cIAP2 (Bertrand et al., 2009), and XIAP (Krieg et al., 2009) take place. This induces conjugation of K63-linked ubiquitin chains on Rip2 (Hasegawa et al., 2008; Yang et al., 2007), reportedly mediated by cIAP1/2 (Bertrand et al., 2009). The Rip2 poly-ubiquitin chains serve as a scaffold to recruit and activate the downstream signaling complexes such as IKK kinase (Hasegawa et al., 2008; Inohara et al., 2000). Recently, it was shown that XIAP ubiquitylates Rip2 after Nod2 stimulation to facilitate the linear ubiquitin chain assembly complex (LUBAC) recruitment, which regulates Nod2-dependent immune signaling (Damgaard et al., 2012), a process abrogated by mutations in XIAP in patients suffering from X-linked lymphoproliferative syndrome type 2 (XLP-2) (Marsh et al., 2010; Rigaud et al., 2006). ITCH, another E3-ligase, is also involved in poly-ubiquitinating Rip2 through K63 linkages although, unlike other E3 ligases, it acts to negatively regulate Rip2-driven NF-κB responses while promoting the MAPK responses (Tao et al., 2009). Similarly, A20, a de-ubiquitinating enzyme, cleaves activating poly-K63-ubiquitin chains from Rip2 leading to an inhibition of NF-κB activation (Hitotsumatsu et al., 2008). Cylindromatosis (CYLD), another de-ubiquitinating enzyme that targets members of the TRAF family and acts as a negative regulator of NF-κB activation (Trompouki et al., 2003), has
recently been shown to be upregulated in a Nod1-dependent manner as part of a subversion mechanism by *Klebsiella pneumoniae* (Regueiro et al., 2011).

Initiation of NF-κB signaling occurs when activation of Rip2 leads to its poly-ubiquitination (Hasegawa et al., 2008; Hitotsumatsu et al., 2008; Krieg et al., 2009; Yang et al., 2007), as well as triggering activation of NFκB essential modulator (NEMO), the regulator of the IKK subunit, to promote activation of the catalytic subunits IKKα and IKKβ (Abbott et al., 2004). In addition, Rip2 mediates the recruitment of transforming growth factor β-activated kinase (TAK1), which is essential for IKK activation (Hasegawa et al., 2008; Kim et al., 2008a). Once activated, IKK phosphorylates the inhibitor IκB targeting it for degradation, thereby releasing NF-κB and allowing it to translocate to the nucleus (Hayden and Ghosh, 2004). Similar to the NF-κB pathway, TAK1 and Rip2 are required for Nod1- and Nod2-mediated MAPK activation, although the link between Nod activation and MAPK activation is less clear (da Silva Correia et al., 2007; Park et al., 2007a; Windheim et al., 2007). Nod activation leads to activation of JNK (Girardin et al., 2001; Hsu et al., 2007; Kim et al., 2008b), ERK1/ERK2 (Kim et al., 2008b; Kobayashi et al., 2005; Windheim et al., 2007), and p38 (Hsu et al., 2007; Kim et al., 2008b; Kobayashi et al., 2005; Opitz et al., 2006). The activation of these MAPK and NF-κB pathways induce the expression of several cytokines, such as IL-6, IL-10, and chemokines, such as IL-8, monocyte chemotactic protein (MCP)-1, and keratinocyte-derived chemokine (KC), as well as antimicrobial peptides, mainly α defensins (Kersse et al., 2011).

Recent reports also provide evidence for additional pathways originating from Nod1 and Nod2. Both Nod1 and Nod2 have been shown to induce type I IFN production (Sabbah et al., 2009; Watanabe et al., 2010a), although the triggers and the molecular mechanisms differ significantly. Nod1 activation is initiated by Nod1 ligand, iE-DAP, leading to downstream signaling involving Rip2 interacting with TRAF3 to induce type 1 IFN production (Watanabe et al., 2010a). On the other hand, Nod2 induces type I IFN production in response to viral ssRNA, through a peptidoglycan-independent, Rip2-independent mechanism involving mitochondrial signaling complex (Sabbah et al., 2009).

Autophagy is a highly conserved recycling process where damaged organelles or pathogens can be degraded through the lysosomal machinery (Deretic and Levine, 2009). Nod1 and Nod2 have also been linked to autophagy signaling, by recruiting ATG16L1, one of the key components of
autophagy machinery, to the plasma membrane at bacterial entry sites (Cooney et al., 2010; Homer et al., 2010; Travassos et al., 2010). The involvement of Rip2 in Nod1/2-mediated autophagy is debatable since one study found this process to be Rip2 independent (Travassos et al., 2010) whereas another group found Rip2 essential for the process (Cooney et al., 2010).

1.2.7 Role of Nod1 and Nod2 in Host defense against pathogens at mucosal surfaces

Mucosal surfaces are the place where epithelial cells come in contact with resident microbiota or potentially harmful pathogens. This is especially true in the case of gut as well as vaginal epithelium as these surfaces experience constant exposure to microorganisms. Pattern recognition receptors such as Nod1 and Nod2 are found within epithelium to help the detection of microorganisms.

1.2.7.1 Role in innate immunity

Nod1 and Nod2 are involved in sensing of numerous pathogenic bacteria. In vitro studies reveal that Nod1 detects *Shigella flexneri* (Girardin et al., 2001), enteroinvasive *Escherichia coli* (Kim et al., 2004), *Chlamydia* (Buchholz and Stephens, 2008; Opitz et al., 2005; Welter-Stahl et al., 2006), *Pseudomonas aeruginosa* (Travassos et al., 2005), *Campylobacter jejuni* (Zilbauer et al., 2007), and *Helicobacter pylori* (Boughan et al., 2006; Viala et al., 2004). Similarly, Nod2 is involved in the recognition of *Listeria monocytogenes* (Kobayashi et al., 2005), *Staphylococcus aureus* (Girardin et al., 2003b), *Streptococcus pneumonia* (Opitz et al., 2004), and *Mycobacterium tuberculosis* (Ferwerda et al., 2005). Although these data support the role of Nod1 and Nod2 in host defense against pathogens in vitro, in vivo evidence using Nod1−/− and Nod2−/− mice is not as abundant. While in vitro infection of primary cells with *Chlamydia trachomatis* suggests Nod1 is involved in detecting the organism, vaginally infected Nod1−/− mice showed normal pathology, suggesting no critical role for Nod1 in vivo (Welter-Stahl et al., 2006). Similarly, Nod2−/− mice was slightly more susceptible to oral infection with *Yersinia pseudotuberculosis* (Meinzer et al., 2008), and yet, no difference was observed in cytokine responses of wild-type and Nod2-deficient macrophages to in vitro infection with *Yersinia* (Kim et al., 2008b). Nevertheless, several in vivo studies have clearly demonstrated a key role for Nod1 and Nod2 in host defense against pathogens such as *H. pylori*, *L. monocytogenes*, and *Staphylococcus aureus* (Hruz et al., 2009; Kobayashi et al., 2005; Viala et al., 2004). For
example, *Nod1*<sup>−/−</sup> mice had higher bacterial loads following intragastric infection with *H. pylori* (Viala et al., 2004), and had impaired expression of the antimicrobial peptide β-defensin 4 (Boughan et al., 2006), suggesting that Nod1 plays an important role in the induction of antimicrobial peptides following infection with this pathogen. Likewise, *Nod2*<sup>−/−</sup> mice infected orally with *L. monocytogenes* were more susceptible to infection, and demonstrated low level of certain antimicrobial α-defensin peptides compared to wildtype controls (Kobayashi et al., 2005).

In addition to inducing antimicrobial peptides, Nod1 and Nod2 induce the production of nitric oxide, a molecule that is known to be directly microbicidal (Magalhaes et al., 2005; Park et al., 2007b; Totemeyer et al., 2006). Moreover, activation of Nod1 and Nod2 by live bacteria triggers pro-inflammatory responses leading to the induction of cytokine and chemokines (Opitz et al., 2005; Opitz et al., 2006). Interestingly, Nod1 was also recently demonstrated to provide protection against the intracellular parasite *Trypanosoma cruzi* (Silva et al., 2010), which is the etiological agent of Chagas disease (Silva et al., 2010), despite the lack of peptidoglycan in this pathogen. *Nod1*-deficient mice were highly susceptible to *T. cruzi* infection in vivo, and it was suggested the underlying mechanism for Nod1-dependent resistance appeared to be independent of cytokine production and dependent on the impaired killing ability of macrophages (Silva et al., 2010).

### 1.2.7.2 Role in adaptive immunity

Nod1 and Nod2 have also been implicated in the induction of adaptive immune responses. Indeed, as mentioned in section 1.1.2, earlier studies identified Nod1 (tri-, tetrapeptides) and Nod2 ligand (MDP) as adjuvants (Adam et al., 1974; Ellouz et al., 1974). Prior to the identification of Nod2 as the sensor for MDP, it was known that MDP was a crucial component of Freund’s Complete Adjuvant (Adam et al., 1981), and its adjuvanticity suggested that Nod2 might play a role in the induction of adaptive immunity. This appears to be true, as the injection of MDP with a model protein ovalbumin (OVA) in mice induces an adaptive response that is completely abrogated in Nod2-deficient animals (Kobayashi et al., 2005). In addition, recent studies have shown that in vivo immunization of OVA with either the Nod1 or Nod2 ligand results in a Th2-skewed antigen-specific adaptive immunity, characterized by IL-4 and IL-5 producing T cells and IgG1 antibodies (Fritz et al., 2006; Magalhaes et al., 2008), providing further evidence of the role of these NLR proteins in adaptive immunity. More recently, Nod2
activation through MDP was shown to induce Th2-driven allergic inflammation in mice by altering the balance between antigen-specific regulatory T cells (CD4⁺Foxp3⁺) and IL-4-producing CD4 T cells (Duan et al., 2010). These studies suggest that Nod2 activation may lead to Th2 immunity, suggesting a possible role of Nod2 in allergic inflammation.

Interestingly, CD4+ T cells express Nod2, and several groups suggested a role for Nod2 in T-cell function (Rahman et al., 2010; Shaw et al., 2009). Shaw et al. observed that Nod2 was involved in host defense to intraperitoneal challenge with Toxoplasma gondii through a mechanism involving CD4⁺ T cells (Shaw et al., 2009). Nod2-deficient T cells were defective in their induction of IL-2, which is necessary for the generation of both Th1 and Th2, and Nod2 was shown to act downstream of the co-stimulatory molecule CD28, leading to IL-2 transcription. This finding, however, was recently challenged by another group, who found no role for Nod2 in the activation and Th1 differentiation of CD4+ T cells (Caetano et al., 2011). On the other hand, recent work from Rahman et al. demonstrated that FoxP3⁺ T regulatory cells (Tregs) express Nod2 and activate NFκB in response to MDP stimulation (Rahman et al., 2010). Moreover, Crohn’s disease patients with Nod2 mutations had fewer lamina propria Tregs (Rahman et al., 2010). Thus, although it is clear that Nod1 and Nod2 contribute to adaptive immunity, it is still not known whether these receptors contribute by having a direct effect on T cells, or indirectly through interaction with antigen presenting cells (APCs). Further studies are required to delineate the precise mechanism by which Nod1 and Nod2 contribute to adaptive immunity.

1.2.7.3 Nod1 and Nod2 as modulators of Th17 response in the gut

Th17 cells are a subset of differentiated CD4+ T helper cells that produce a group of distinct cytokines including IL-17, and IL-22, which participate in inducing tissue inflammatory response (Korn et al., 2009). The Th17 response plays a crucial role in mucosal immunity to enteric bacterial pathogens. In the gastrointestinal tract, IL-17 and IL-22 were shown to confer protection against various enteric pathogens such as Helicobacter pylori, Citrobacter rodentium and Salmonella enterica serovar Typhimurium (Raffatellu et al., 2008; Velin et al., 2009) (Godinez et al., 2009; Raffatellu et al., 2009). For example, Salmonella enterica serovar Typhimurium induces an acute inflammatory response in the cecum of mice, with early (24-48h) production of IL-17 by γδT cells (Godinez et al., 2008; Godinez et al., 2009; Raffatellu et al., 2009). Interestingly, a recent work by our group identified redundant but critical roles for Nod1
and Nod2 in driving innate Th17 responses during early stages of infection with enteric bacterial pathogens (Geddes et al., 2011). The authors found that mice deficient for both Nod1 and Nod2 showed a blunted early inflammation in both the S. Typhimurium and C. rodentium colitis models, with significantly reduced amounts of IL-17A, and IL-22 in cecal tissue during early infection. The contribution of Nod1 and Nod2 to host protection was achieved through the induction of IL-17A and IL-22, which were produced by CD4+ TCRβ+ Th17 cells in the cecal lamina propria at early times post infection. In addition, Nod expression by both myeloid and somatic cells was required, and dendritic cells were identified as a major source for Nod1/Nod2 dependent production of IL-6, which seemed to be central to this “innate Th17” (iTh17) response. Finally, the presence of the microbiota was essential for priming iTh17 cells, as germ-free mice showed no iTh17 response. Taken together, these findings highlight that Nod1 and Nod2 are critical in the innate activation of Th17 response during early stages of bacterial colitis.

1.3 Peptidoglycan recognition proteins as sensors of peptidoglycan

Aside from Nod1 and Nod2, several other mammalian molecules recognize peptidoglycan including soluble CD14, C-type lectins, lysozymes and peptidoglycan recognition proteins (PGRPs). Among these, PGRPs were first identified in the haemolymph of a silkworm (Bombyx mori) as proteins that could bind to bacterial peptidoglycan and activate antimicrobial host defense mechanism in insects (Yoshida et al., 1996). The cloning of PGRP genes from the fruitfly, Drosophila melanogaster, led to the discovery of mouse and human PGRP orthologs (Kang et al., 1998), which indicated that PGRPs were highly conserved from insects to mammals. PGRPs from D. melanogaster are by far the most extensively studied PGRPs where the central role of these proteins in innate immunity was demonstrated.

1.3.1 PGRPs in Drosophila innate immune system

D. melanogaster does not have an adaptive immunity, and relies heavily on their PGRPs to detect bacterial and fungal pathogens. D. melanogaster has 13 PGRP genes that are transcribed into 19 proteins (Kang et al., 1998; Werner et al., 2000). These proteins can be classified into two subclasses; one subclass of PGRPs can bind and recognize peptidoglycan triggering host defense pathways that generate antibacterial products, whereas the other subclass can hydrolyze pro-inflammatory peptidoglycan and thereby reducing inflammation. PGRPs such as PGRP-SA,
PGRP-SD, PGRP-LC and PGRP-LE fall into the PGRP subclasses involved in peptidoglycan recognition, but not hydrolysis. The subclass of PGRPs that displays N-acetylmuramoyl-L-alanine amidase enzymatic activity includes PGRP-SC1, PGRP-SC2, PGRP-SB1/2, and PGRP-LB (TANJI and Ip, 2005). These proteins participate in two distinct signaling pathways: while the Toll pathway responds to Gram-positive bacteria and fungal pathogens, the IMD (immune deficiency) pathway responds to Gram-negative bacteria. Detection of Gram-positive bacteria upstream of the Toll pathway involves a specific PGRP, PGRP-SA, as its mutant form was impaired in its ability to activate the Toll pathway, and was highly susceptible to infection by Gram-positive bacteria (Michel et al., 2001). Similarly, studies demonstrated that PGRP-LC is the main transmembrane receptor that activates the IMD pathway, since a mutation in the PGRP-LC gene hinders activation of the IMD pathway, rendering the fly highly susceptible to infection with Gram-negative bacteria specifically (Choe et al., 2002; Gottar et al., 2002; Ramet et al., 2002). Moreover, it was later identified that PGRPs in *D. melanogaster* detect specific forms of peptidoglycan; Lys-type peptidoglycan in Gram-positive bacteria favors interaction with PGRP-SA, and activates Toll, whereas *meso*DAP in Gram-negative peptidoglycan leads to activation of the IMD pathway through an interaction with PGRP-LC (Leulier et al., 2003).

1.3.2 *Drosophila* hydrolytic PGRPs in the gut innate immunity

Although the key role of non-hydrolytic PGRPs in innate immunity was demonstrated early on, the function of hydrolytic PGRPs was unknown for many years. Mellroth and colleagues performed the first in vitro study to demonstrate that one of the hydrolytic PGRPs, PGRP-SC1B, hydrolyzed Gram-negative peptidoglycan, resulting in a digested molecule that no longer triggered activation of immune system (Mellroth et al., 2003). Based on this finding, the authors postulated that hydrolytic PGRPs may play a role as scavenger molecules, preventing the activation of inflammatory pathways. Supporting this, others demonstrated that GlcNAc-AnhydroMurNAc-TetraDAP (also known as TCT or tracheal cytotoxin), the minimal peptidoglycan motif that fully activated IMD pathway, became completely inactive in its TetraDAP form (the peptidic product released by hydrolytic PGRPs) (Kaneko et al., 2004; Stenbak et al., 2004). An in vivo validation of the above findings came with a study by Zaidman-Remy and colleagues who demonstrated the key role of an amidase, PGRP-LB, using RNA interference (RNAi) technology to specifically knock down expression of PGRP-LB in adult flies (Zaidman-Remy et al., 2006). This group found that PGPR-LB knockdown flies exhibited
higher expression of the IMD-specific antimicrobial peptides diptericin in response to bacteria and this response was also lasting longer compared to that of wild-type flies. These findings suggest that PGRP-LB acts as a scavenger molecule to down-regulate the IMD pathway by degrading peptidoglycan. Interestingly, the study also revealed that PGRP-LB was important in controlling IMD pathway activation both in the gut and systemically during homeostasis as well as during bacterial infection; at homeostatic level, PGRP-LB knockdown files had enhanced expression of local antimicrobial peptide in the gut, indicating that peptidoglycan fragments released by bacteria present in the digestive tract is normally hydrolyzed by PGRP-LB to inhibit IMD activation. Further, PGPR-LB was shown to prevent systemic immune responses following oral challenge of Gram-negative bacteria or peptidoglycan. All together, this study demonstrated that PGRP-LB has not only a scavenger function in vivo, but also a role as a master regulator of immune homeostasis at epithelial surfaces. Despite the fact that the role of PGRP-LB in regulation of the IMD pathway is clear, the function for other hydrolytic PGRPs, such as PGRP-SCs and PGRP-SB1 and SB2, is not clear. There is conflicting evidence for roles of PGRP-SCs (Bischoff et al., 2006; Garver et al., 2006) and no overt phenotype was shown in PGRP-SB1 and SB2 mutant flies (Zaidman-Remy et al., 2011). Recent study on a systematic analysis of amidase PGRP using gene deletions still support the role of PGRP-LB as a negative regulator of the IMD pathway both locally and systemically (Paredes et al., 2011). Moreover, this group identified a new role of PGRP-LB in downregulating the IMD pathway triggered by commensals in the adult gut, under unchallenged conditions. Further, PGRP-SC family seems to have a role in negatively regulating the IMD pathway during systemic infection only and synergizes with PGRP-LB in the systemic immune response to ingested bacteria (Paredes et al., 2011). The authors also concluded that PGRP-SB1 and SB2 do not seem to play a major role in the regulation of the IMD pathway. Nevertheless, these in vivo studies firmly established that some of these amidases are important to down-modulate the IMD pathway, both systemically and in the gut (Bischoff et al., 2006; Zaidman-Remy et al., 2006).

1.3.3 Mammalian PGRPs

Mammalian PGRPs consist of four members, namely PGLYRP1, PGLYRP2, PGLYRP3, and PGLYRP4, which were initially named PGRP-S, -L, Iα, and Iβ, respectively (on the basis of their short (S), long (L), and intermediate (I) transcript lengths). Three of them (PGLYRP1, PGLYRP3 and PGLYRP4) are known to have bactericidal properties against both Gram-positive
and Gram-negative bacteria, but no known amidase activity, whereas PGLYRP2 is a peptidoglycan-hydrolytic enzyme known to have the N-acetylmuramoyl-L-alanine amidase activity, which is the only conserved common function of insect and mammalian PGRPs (Royet et al., 2011).

1.3.3.1 Structure

Mammalian PGRPs have at least one C-terminal PGRP domain that comprises approximately 165 amino acids. PGRP domain is structurally homologous to bacteriophage and bacterial type 2 N-acetylmuramoyl-L-alanine amidases (Dziarski and Gupta, 2006; Kang et al., 1998; Kiselev et al., 1998; Liu et al., 2001), which suggests that PGRPs and prokaryotic type 2 amidases may have evolved from the same ancestral gene. Mammalian PGLYRP1 is ~200 amino acids long, has one PGRP domain and a molecular mass of 18-20 kDa (Dziarski and Gupta, 2006) (Figure 1.6a). PGLYRP2 has one C-terminal PGRP domain, a molecular mass of 64-80 kDa, and a very long unique N-terminal sequence that does not resemble PGRP domain or any other proteins (Zhang et al., 2005). PGLYRP3 and PGLYRP4 both have two PGRP domains, and a molecular mass of ~35 kDa and ~40 kDa, respectively (Royet et al., 2011).

Initially, mammalian PGRPs were thought to be transmembrane proteins due to their predicted transmembrane domains (Liu et al., 2001; Wang et al., 2003), which were later found to be hydrophobic domains (Zhang et al., 2005), likely with some other function. Indeed, all mammalian PGRPs are secreted (Guan et al., 2004; Zhang et al., 2005) and, in the case of PGLYRP1, PGLYRP3, and PGLYRP4, form disulfide-linked homodimers (Lu et al., 2006; Xu et al., 2004; Zhang et al., 2005). In addition, PGLYRP3 and PGLYRP4 almost always form disulfide-linked heterodimers if they are expressed in the same cells (Lu et al., 2006). On the other hand, PGLYRP2 form dimers that are not disulfide-linked (De Pauw et al., 1995).

The crystal structure of PGRPs reveals that the general structural design of the PGRP domain is similar to type 2 bacteriophage amidases, which has three α-helices and several central β-sheet strands (Guan et al., 2004; Kim et al., 2003). PGRP domain contains a peptidoglycan-binding groove, and a PGRP-specific segment, not present in type 2 amidases (Dziarski and Gupta, 2006; Kim et al., 2003; Royet and Dziarski, 2007). The peptidoglycan-binding groove of PGLYRP2 has a Zn\textsuperscript{2+}-binding site, which consists of conserved amino acids including one Cys (C530) residue critical for Zn\textsuperscript{2+}-binding and amidase activity. This Cys is substituted with Ser in the
A. The figure shows diagrams of the domain structure of mammalian PGRPs (adapted from *Nature Reviews Immunology* (Royet et al., 2011)).

B. PGLYRP2 and lysozyme are the two unique host enzymes known to hydrolyze peptidoglycan. Lysozyme cleaves the sugar group between MurNAc (M) and GlcNAc (G), while PGLYRP2 hydrolyzes the lactyl bond between M and the peptides.

Figure 1.6 (A) The structure of mammalian PGRPs, (B) The host peptidoglycan hydrolases PGLYRP2 and lysozyme.
catalytic site in PGLYRP1, PGLYRP3, and PGLYRP4 (Gelius et al., 2003; Wang et al., 2003) and thus, these PGRPs do not have amidase activity.

1.3.3.2 Peptidoglycan binding

Based on the crystal structure of PGRPs, peptidoglycan-binding groove in PGRP domain is shown to be specific for muramyl tripeptide (Chang et al., 2004; Guan et al., 2004; Kim et al., 2003), whereas it does not bind an MDP or a peptide without MurNAc (Guan et al., 2006; Kumar et al., 2005; Swaminathan et al., 2006). It binds muramyl tetrapeptide and muramyl pentapeptide with higher affinity (Kumar et al., 2005; Swaminathan et al., 2006). Indeed, the crystal structure of C-terminal PGRP domain of PGLYRP3 indicates that binding of muramyl pentapeptide to this region induces a conformational change in the PGRP domain locking the ligand in the binding groove (Guan et al., 2006).

Mammalian PGRPs bind to both Gram-positive and Gram-negative bacteria and some fungi (Lu et al., 2006; Tydell et al., 2006). Crystallographic studies also suggest that these PGPRs can discriminate between Lys-type and DAP-type peptidoglycans (Chang et al., 2006; Kumar et al., 2005; Lim et al., 2006; Swaminathan et al., 2006). For example, human PGLYRP3 was shown to bind Lys-type peptidoglycan with higher affinity than DAP-type peptidoglycan, whereas human PGLYRP1 was shown to favor DAP-type peptidoglycan over Lys-type. Although human and mouse PGRPs have a high affinity for peptidoglycan, some mammalian PGRPs such as bovine PGLYRP1 have been shown to bind other microbial molecules, including LPS and LTA with high affinity (Tydell et al., 2006). Moreover, bovine PGLYRP1 and human PGLYRP3 and PGLYRP4 can also bind fungi-derived molecules (Lu et al., 2006; Tydell et al., 2006).

1.3.3.3 Amidase activity

There are two known host peptidoglycan-degrading enzymes in mammals. The first, lysozyme, has been studied extensively since its discovery 90 years ago, and is known for its role as an antimicrobial molecule (Fleming, 1922). Lysozyme is found in the serum, body fluid (such as tears, sweat, saliva), the granules of neutrophils, and intestinal paneth cells (Chipman and Sharon, 1969; Ganz et al., 2003). Lysozyme cleaves the β-1-4 linkage between the N-acetylmuramic acid (MurNAc) and the N-acetylgulosamine (GlcNAc) of peptidoglycan, releasing glycopeptides. The other enzyme, PGLYRP2, cleaves the lactyl bond between the
MurNAc and L-Ala in bacterial peptidoglycan (Figure 1.6b). PGLYRP2 is constitutively expressed in the liver, and secreted into bloodstream (Wang et al., 2003). Indeed, PGLYRP2 from the liver was identified to be the same enzyme as serum N-acetylmuramoyl-L-alanine amidase which was identified earlier but, at the time, was not cloned (Zhang et al., 2005). Moreover, PGLYRP2 not only is expressed in various parts of oral cavities and intestinal epithelial cells (Saha et al., 2010) but also its expression is induced in keratinocytes and other epithelial cells and fibroblasts by exposure to bacteria and cytokines (Li et al., 2006; Saha et al., 2009; Uehara et al., 2005; Wang et al., 2005). In addition, Pglyrp2 expression was recently characterized in small intestinal intraepithelial leukocytes (IELs), with the majority of its expression in T lymphocytes (Duerr et al., 2010).

In contrast to the other three mammalian PGRPs, PGLYRP2 does not function as an antimicrobial protein (Gelius et al., 2003). Instead, it hydrolyzes the bond between the sugar group and the peptide stem in peptidoglycan. The minimum peptidoglycan fragment hydrolyzed by PGLYRP2 is muramyl tripeptide (containing either mesoDAP or Lys), similar to the minimal PGRP-binding fragment (Chang et al., 2004; Guan et al., 2004; Kim et al., 2003). MDP, a typical Nod2 ligand, is therefore not cleaved by PGLYRP2 (Wang et al., 2003). Serum PGLYRP2 might function as a scavenger molecule similar to amidase-active insect PGRPs, as it was shown to inactivate inflammatory peptidoglycan by lowering its cytokine-inducing properties (Hoijer et al., 1997b). However, in tissues, PGLYRP2 seems to participate in induction of inflammatory response, although this response is independent of its amidase and peptidoglycan-binding activities (Saha et al., 2009). Thus, the role of the amidase-active mammalian PGLYRP2 remains unclear and will be discussed in detail in chapter 5.

1.3.3.4 Bactericidal activity

PGLYRP1 is highly expressed in polymorphonuclear leukocyte (PMNs) granules, especially in the secretory granules of neutrophils (Tydell et al., 2002). PGLYRP1 is also found in intestinal M (microfold) cells (Lo et al., 2003) and in many non-immune cells, such as epithelial cells and fibroblasts (Saha et al., 2009; Uehara et al., 2005). PGLYRP3 and PGLYRP4 proteins are selectively expressed in tissues that come into contact with the environment (Lu et al., 2006; Mathur et al., 2004), such as skin, eyes, oral cavity and intestinal tract. The expression of PGLYRP3 and PGLYRP4 were shown to be up-regulated by bacteria and their products in
keratinocytes (Lu et al., 2006), fibroblasts (Saha et al., 2009), and oral epithelial cells (Uehara et al., 2005).

PGLYRP1, PGLYRP3, PGLYRP4 are bactericidal or bacteriostatic for many pathogenic and non-pathogenic Gram-positive and Gram-negative bacteria (Lu et al., 2006; Wang et al., 2007) in vitro. In vivo, *Pglyrp1*-deficient mice were found to be susceptible to infections with some Gram-positive bacteria (Dziarski et al., 2003). Similarly, using recombinant PGLYRP3 in wild-type mice, PGLYRP3 was shown to play a bactericidal role, thus preventing *S. aureus* lung infection in mice (Lu et al., 2006).

Interestingly, these mammalian bactericidal proteins have very distinct characteristics that separate them from the currently known mammalian antimicrobial peptides. Indeed, mammalian constitute a new class of bactericidal proteins; PGRPs are much larger than vertebrate antimicrobial peptides; PGLYRPs are disulfide-linked dimers, 44-115kDa in size (Lu et al., 2006), whereas vertebrate antimicrobial peptides are typically 3-15kDa. The expression patterns of mammalian PGRPs and antimicrobial peptides are different, and mammalian PGRPs are not expressed by some cells that produce large amounts of antimicrobial peptides such as Paneth cells (Ganz et al., 2003; Lu et al., 2006; Zasloff, 2002). Moreover, PGRPs kill bacteria probably by interacting with peptidoglycan whereas antimicrobial peptides kill bacteria by permeabilizing bacterial membranes (Ganz et al., 2003; Zasloff, 2002).

The mechanism underlying bacterial killing by mammalian PGRPs was completely unknown until recently, although it was previously proposed that PGPRs kill bacteria by inhibiting peptidoglycan synthesis (Dziarski and Gupta, 2010). Kashyap and colleagues recently demonstrated that PGPRs kill bacteria by exploiting bacterial two-component stress response systems, designed to respond quickly to environmental stress. If stress is sustained or its level is too high, the bacterial two-component systems will be over-activated and bacteria will die. Thus, the mammalian PGRPs trigger this bacterial defense/suicide mechanism to kill bacteria (Kashyap et al., 2011).
1.3.3.5 Role of mammalian PGRPs in innate immunity

As discussed above, *Drosophila* PGRPs have many functions in the host response to infection, mostly involved in activating or downregulating their immune pathways following microbial sensing (Royet and Dziarski, 2007). In contrast, the role of mammalian PGRPs remains largely unknown, especially in vivo, despite the fact that PGRPs specifically bind bacterial peptidoglycan and that some of them display antibacterial activity in vitro (Lu et al., 2006; Tydell et al., 2002; Tydell et al., 2006; Wang et al., 2007).

Initial study with *Pglyrp2*-deficient mice showed a normal response intraperitoneal infection with Gram-positive or Gram-negative bacteria (Xu et al., 2004), suggesting no role for PGLYRP2 in mammalian immunity. Subsequently, PGLYRP2 was shown to play a role in local tissue inflammation and acute arthritis: because insect amidase-active PGRPs have anti-inflammatory properties and hydrolyze pro-inflammatory peptidoglycan, Saha and colleagues tested whether amidase active PGLYRP2 played a similar anti-inflammatory role in vivo in a peptidoglycan-induced arthritis model (Saha et al., 2009). Surprisingly, this group found that PGLYRP2 was required for the induction of peptidoglycan-induced local inflammation and arthritis as *Pglyrp2*-deficient mice were resistant to peptidoglycan-induced arthritis. Moreover, this phenotype was dependent on Nod2, and independent of PGLYRP2 amidase activity. Interestingly, the other three mammalian PGRPs were shown to have an opposite anti-inflammatory effect in the same model, with *Pglyrp1*-deficient mice exhibiting arthritis of the highest severity. Moreover, although the protein is secreted, the effect of PGLYRP2 was local in the site of injection and not systemic.

Recently, the same group further demonstrated that all four mammalian PGRPs were implicated in host protection in a dextran sulfate sodium (DSS)-induced colitis model (Saha et al., 2010). Using all four individual PGRP knockout mice, they showed that these mice displayed relatively similar increased sensitivity to DSS and altered immune responses as compared to wild-type mice. This increased sensitivity to DSS-induced colitis was due to the presence of a more inflammatory gut microbiota, higher production of interferon-γ, and an increased number of NK cells in the colon. This finding perhaps suggests a role of mammalian PGRPs in the maintenance of proper gut homeostasis by having a direct effect on the microbiota composition. More studies
will be required to delineate the exact mechanism through which individual PGPRs confer protection in mucosal immunity.

### 1.4 Mucosal biology in the gut

#### 1.4.1 Gut overview – intestinal epithelium

The human intestinal tract is continuously exposed to a wide range of food antigens and other antigens from the external environment. Moreover, unlike epithelial surfaces such as the lung or bladder, which are sterile under non-pathological conditions, the gut epithelium harbors the greatest number of resident bacteria (commensals) in the body, with $10^{11}$ (per gram of intestinal content) bacteria in the large intestine (Ley et al., 2006). Thus, the gut epithelium has a formidable task to maintain a physical and immunological barrier, especially in the presence of enormous quantities of luminal bacteria. The innate immune system at the gut barrier must keep a fine balance between the need to tolerate the potentially pro-inflammatory commensals in the lumen and the need to protect the host from invading enteric pathogens. This is achieved by components of the innate sensing system such as NLRs and TLRs present at the intestinal barrier in various cell types where they influence every level of the immune response.

*Physical barriers*

The physical barrier of intestinal mucosal surface is maintained by complex mechanism acting on several levels. The intestinal epithelial cells (IECs) are a single layer of interconnected, polarized cells, composed of several specialized cell subsets including M cells, goblet cells, Paneth cells, and columnar epithelial cells (Goto and Kiyono, 2012) (Figure 1.7). Goblet cells are secretory epithelial cells that produce mucins, which are highly glycosylated macromolecules that form the mucus, a gel-like physical barrier between the gut contents and epithelial cells (McGuckin et al., 2011). Paneth cells, another type of secretory epithelial cells, are specific to the small intestine at the basal sites of crypts, although Paneth cell-related cells are recently observed in colon crypt region (Sato et al., 2011). These cells produce antimicrobial peptides such as defensins, lysozymes, and RegIIIγ (Mukherjee et al., 2008), which contribute to keeping the crypts sterile of commensals and enteric pathogens. The production of antimicrobial peptide is regulated by several PRMs, including TLR adaptor, MyD88, and Nod2 (Kobayashi et al., 2005; Vaishnava et al., 2008). For example, Nod2 is prominently expressed in Paneth cells.
The intestinal epithelial cells consist of several specialized cells including M cells, goblet cells, Paneth cells, and columnar epithelial cells. M cells sample antigens within the lumen and deliver these antigens to the initiation compartment of the organized lymphoid tissues, namely the Peyer’s patches and the mesenteric lymph nodes (MLNs). Goblet cells secrete mucins, which provide a physical barrier between the gut contents and epithelial cells. Paneth cells are typically found in the crypts of the small intestine, and secrete antimicrobial peptides, contributing to keep the crypts sterile. Intestinal lamina propria lymphocytes (LPLs) are found scattered within the subepithelial lamina propria (LP), and the intraepithelial lymphocytes (IELs) are embedded in the epithelial-cell layer.
and the expression of defensins by Paneth cells is impaired in Nod2-deficient mice (Kobayashi et al., 2005; Petnicki-Ocwieja et al., 2009). M cells predominantly reside in the regions of follicle-associated epithelium (FAE) covering Peyer’s Patches (PPs), and act to sample antigens within the lumen and present antigens to APCs initiating mucosal immune responses (Hathaway and Kraehenbuhl, 2000).

**Immunological barriers**

With regards to immune function, the intestinal structures that contain lymphocytes are typically divided into three different anatomical compartments. The gut-associated lymphoid tissue (GALT) includes the mesenteric lymph nodes (MLNs), the PPs of the small intestine and similar follicular aggregates of the large intestine and cecum (Cheroutre and Madakamutil, 2004). The lamina propria (LP), a connective tissue that underlines the epithelium of the mucosa, contains various myeloid and lymphoid cells, including macrophages, dendritic cells, T cells and B cells (Cheroutre et al., 2011). Lymphocytes localized within the subepithelial LP are called lamina propria lymphocytes (LPLs) (Figure 1.7). Additionally, numerous IELs reside on the other side of the basement membrane from the LP, interspersed among the luminal epithelial cells (Figure 1.7).

**IELs/LPLs**

T cells residing in the GALT have the most in common with peripheral T cells (Cheroutre et al., 2011). On the other hand, intestinal LPLs and IELs display a number of unique characteristics that distinguish them from conventional T cell pools in the periphery. IELs comprise mostly T cells belonging to both the T cell receptor (TCR)-γδ and TCR-αβ lineages (Cheroutre et al., 2011). Unlike conventional T cells, however, IELs are antigen-experienced T cells that do not require priming, and immediately release cytokines and cytotoxic granules to kill infected cells (Cheroutre et al., 2011). These IELs typically express activation markers, such as CD44 and CD69, as well as CD103 (also known as the αE integrin), which interacts with E-cadherin on intestinal epithelial cells (Cepek et al., 1994; Kilshaw and Murant, 1990). Furthermore, most IELs express effector cytokines, such as IFN-γ, IL-2, IL-4 or IL-17 (Cheroutre et al., 2011). IELs are extremely heterogeneous with regard to their function and phenotype and are represented by conventional and nonconventional T cell subsets. Many TCR-αβ IELs express CD8αα together
with CD4 or CD8αβ, however, a large fraction expresses CD8αα alone (Guy-Grand et al., 1991). In contrast to TCR-γδ T cells located in lymphoid tissues that predominantly lack CD8 expression, the vast majority of TCR-γδ IELs express the CD8αα co-receptor (Leishman et al., 2001; Pardigon et al., 2004).

Similar to IELs, LPLs are antigen-experienced T cells that mostly express TCR-αβ together with one of the co-receptors, CD4 or CD8αβ. In the small intestine, LPLs express more CD8αβ whereas LPLs of the large intestine is slightly skewed towards CD4 expression (Cheroutre and Madakamutil, 2004). These LPLs also have unique characteristics that set them apart from the conventional T cells (Kim et al., 1999; Masopust et al., 2001; Zeitz et al., 1991; Zeitz et al., 1994). Together, the distinct nature and complexity of these IELs/LPLs is well suited for coping with infections while simultaneously maintaining tolerance to innocuous antigens from the diet or from non-invasive resident bacteria at the critical interface between the intestinal epithelial barrier and the gut lumen.

### 1.4.2 Inflammatory Bowel Disease

Crohn’s disease (CD) and ulcerative colitis (UC) make up inflammatory bowel disease (IBD), and these represent lifelong chronic, relapsing inflammatory conditions that can manifest as disease restricted to the colonic mucosa in UC patients, or disease affecting the mucosa, epithelium, and mucosal layer throughout the entire gastrointestinal tract in CD patients (Baumgart and Sandborn, 2007). Although the etiology of IBD is not completely understood, it is believed to involve genetic, immunological, environmental and microbial aspects (Kaser et al., 2010). IBD have been intensively studied genetically, including genome-wide association studies (GWAS). Indeed, recent GWAS reveal 99 unique IBD susceptibility associations, 71 for CD and 47 for UC (Anderson et al., 2011; Franke et al., 2010; Rivas et al., 2011; Van Limbergen et al., 2011).

Among a number of NLR family members whose genes have been identified as susceptibility loci for CD, *Nod2* was the first identified susceptibility gene for CD within an IBD susceptibility locus on chromosome 16 (Hugot et al., 2001; Ogura et al., 2001), and is the locus most strongly linked to the disorder. A recent GWAS revealed five new rare variants of *Nod2* (R311W, S431L, R703C, N852S and M863V) at higher frequencies in CD patients versus controls and UC
patients, two of which displayed impaired localization to the membrane and MDP-driven NF-κB activation (Rivas et al., 2011). Unlike the results obtained for Nod2, studies that were conducted to investigate the association of Nod1 polymorphism with IBD reported conflicting results (Franke et al., 2006; Lu et al., 2010; McGovern et al., 2005; Van Limbergen et al., 2007).

The altered composition of the microbiota, also known as dysbiosis, is a hallmark of IBD. The importance of the microbiota in IBD has been highlighted as several studies in human patients with IBD have shown severely altered intestinal microbial community structure, compared to healthy controls (Eckburg and Relman, 2007; Frank et al., 2007; Ott et al., 2004). Moreover, the function of a number of genes associated with IBD could affect microbial community structure and predispose to inflammation. Indeed, Nod2 was recently found to be an important regulator of the commensal gut microbiota in mice (Petnicki-Ocwieja et al., 2009; Rehman et al., 2011). Nod2-deficient mice display diminished bacterial killing ability, increased loads of commensal bacteria and are less effective in preventing the colonization of pathogenic bacteria (Petnicki-Ocwieja et al., 2009). Further, compared with wild-type mice, Nod2-deficient mice display substantially elevated numbers of Firmicutes and Bacteroidetes, which are the two main commensal phyla in the gut microbiota (Petnicki-Ocwieja et al., 2009; Rehman et al., 2011). Interestingly, a similar shift in commensal phyla is observed in CD patients homozygous for the Nod2 frameshift (FS) mutation (Rehman et al., 2011), in that these Nod2 FS CD patients had increased abundance of the phyla Firmicutes and Bacteroidetes.

Dysbiosis in IBD has also been linked to the altered expression of Paneth cell defensins in patients with CD in relation to Nod2 genotype. Nod2-deficient mice show a blunted production of α-defensin in Paneth cells, suggesting that murine Nod2 is required for the intestinal expression of antimicrobial peptides (Kobayashi et al., 2005). Consistent with these data, the expression of α-defensin in Paneth cells is impaired in CD patients carrying the Nod2 mutation (Wehkamp et al., 2004). The same group demonstrated that the reduced expression of the α-defensin, HD5, was linked to the Nod2 FS mutation (Bevins et al., 2009; Wehkamp et al., 2005), although this finding was challenged by another group (Simms et al., 2008). Despite the controversy, these findings may suggest a possible mechanism by which the Nod2 mutation affects the production of antimicrobial peptides in Paneth cells, thereby predisposing the host to the development of the CD as a result of the colonization of aberrant microbiota.
1.4.3 Animal models of inflammatory bowel disease

Over the years, several animal models of colitis, including chemically induced models, and bacterially induced models have been generated to study IBD. Although no animal model can completely recreate the full spectrum of intestinal pathology observed in either CD or UC, these models can exhibit some of the pathogenic hallmarks of IBD. The most commonly used chemical models are the DSS and trinitrobenzene sulfonic acid (TNBS)-induced models, where the chemical insult damages the epithelial layer leading to acute infiltration of inflammatory cells. Two intestinal infection models, employing *Citrobacter rodentium* in normal mice and *Salmonella enterica* serovar Typhimurium in antibiotic-pretreated mice, have been widely used over the last few years, because infections are robust, and cause significant intestinal inflammation (Eckmann, 2006). The next section will provide some insights into the streptomycin-treated *Salmonella* colitis model, which will also be covered in detail in Chapter 6.

1.4.3.1 *Salmonella enterica* serovar Typhimurium

*Salmonella* is a Gram-negative bacterium in the family of the Enterobacteriaceae (Kaiser et al., 2012). Although many different species exist, *Salmonella* are classified into two major groups: *Salmonella typhi* is responsible for a systemic invasive disease (typhoid fever) in humans, and it does not colonize mice by oral challenge. Non-typhoid *Salmonella*, such as *Salmonella enterica* serovar Typhimurium, evokes nonsystemic gastroenteritis in humans and cattle, a diarrheal disease characterized by acute neutrophilic intestinal inflammation (Eckmann, 2006). In contrast, oral infection of mice with these bacteria causes no major intestinal disease, but rather systemic infection resembling typhoid fever in humans (Santos et al., 2001). This colonization resistance to *Salmonella* enteropathogenesis can be partly overcome by treating mice with an oral antibiotic, such as streptomycin or kanamycin (Endt et al., 2010; Sekirov et al., 2008), before oral infection with the organism engineered to become resistant to the particular antibiotic. The antibiotics diminish the normal intestinal microbiota, likely allowing the inoculated *Salmonella* to colonize the lumen of cecum and colon more effectively than without antibiotic conditioning. Streptomycin treatment leads to a transient clearance of the normal intestinal microbiota (>10-fold reduced in density and composition) (Stecher et al., 2007), allowing the orally inoculated streptomycin-resistant *Salmonella* to colonize the cecum and colon (10^8 – 10^{10} cfu/g stool) effectively (Bohnhoff et al., 1964a, b). Consequently, *Salmonella* establishes intestinal infection, which is accompanied by marked mucosal inflammation within the first day after oral infection.
Similar results have been obtained after infection of germ-free mice with *S. typhimurium*, underlining the importance of the normal microbiota in limiting *Salmonella*-induced intestinal inflammation in mice (Stecher et al., 2005).

Intestinal inflammation in this streptomycin-treated *Salmonella* mouse model is characterized by rapid crypt loss, epithelial erosions and goblet cell loss, mucosal and submucosal infiltration with acute inflammatory cells, particularly neutrophils, and marked edema, in the cecum and, to a lesser degree, in the colon (Barthel et al., 2003; Coombes et al., 2005). This model has been useful for dissecting both bacterial and host factors that modulate inflammation (Hapfelmeier and Hardt, 2005), especially in the early (4-72h) intestinal events that occur after oral *Salmonella* infection. For example, the adaptor protein MyD88, which is required for signaling through most TLRs (Hapfelmeier et al., 2005), was shown to be important for inducing colitis after infection with *S. Typhimurium* mutant strain lacking the *Salmonella* pathogenicity island 2 (SPI-2)-encoded type III secretion systems (TTSS), but not those lacking the SPI-1 system (Hapfelmeier et al., 2005). Previously, our group and others showed that host responses to *Salmonella* infection are influenced by Nod1 and Nod2 (Hisamatsu et al., 2003; Le Bourhis et al., 2009) although these reports did not provide in-depth analysis of the role of Nod1/Nod2 in mediating inflammation during *Salmonella* colitis. Recently, a closer examination for a role of Nod1 and Nod2 by our group revealed that mice lacking both Nod1 and Nod2 (Nod double knockout (NodDKO)) have reduced cecal inflammation by histopathology and cytokine production, coinciding with an increased bacterial burden (Geddes et al., 2010). Furthermore, Nod1 and Nod2 signaling from both hematopoietic and nonhematopoietic cells contributed to the inflammatory response to *Salmonella* infection. In agreement with our previous findings (Le Bourhis et al., 2009), the authors also demonstrated that Rip2, the adaptor protein for both Nod1 and Nod2 signaling, was critical during infection with *Salmonella* cultured under SPI-2-inducing conditions, but not SPI-1-inducing conditions. Thus, these findings support a role for Nod proteins in the responses to infection with *Salmonella enterica* serovar Typhimurium.
Chapter 2
Thesis Objectives

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The goal of my thesis was to provide in depth examination of cellular or secreted innate immune molecules that recognize bacterial peptidoglycan. An important part of the thesis focused on the intracellular innate immune receptors, Nod1 and Nod2, with a closer look at their common signaling component, Rip2, as well as at the mechanism by which Nod1 or Nod2 ligand enters the epithelial cells. The objective for the last section was to examine the in vivo role of PGLYRP2, a secreted peptidoglycan recognition protein, in a murine bacterial colitis model, and further, investigate its relationship with Nod1 and Nod2 in this model.

My PhD thesis addressed three specific aims:

1. Investigate the role of Rip2, the common adaptor protein in Nod1 and Nod2 signaling, in modulating adaptive immunity triggered by Nod1 and Nod2 ligands in vivo (Chapter 3).

2. Determine the entry mechanisms of Nod1 and Nod2 ligands into epithelial cells using in vitro biochemical assays (Chapter 4). Further, design synthetically labeled muramyl peptides to track their trafficking within cells by immunofluorescence (Chapter 5).

3. Determine the role of mouse peptidoglycan recognition protein, PGLYRP2, in the innate immune response to Salmonella enterica serovar Typhimurium infection in vivo (Chapter 6).
Chapter 3
Essential Role of Rip2 in the modulation of innate and adaptive immunity triggered by Nod1 and Nod2 ligands

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* These authors contributed equally to the experimental design of this research and the writing of the publication.

Contribution of Data

All experiments/analyses were performed by Jooeun Lee, unless noted otherwise;

Figure 3.1, Figure 3.4, and 3.6 – the quantitative data were provided by Joao G. Magalhaes.
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3.1 Abstract

Muramyl peptides are the building blocks of bacterial peptidoglycan, and their biological functions in mammals have been extensively studied. In particular, muramyl peptides trigger inflammation, contribute to host defense against microbial infections, and modulate the adaptive immune response to antigens. These bacterial molecules are detected by Nod1 and Nod2, and recent evidence suggests that muramyl dipeptide also activates NLRP3 and NLRP1 inflammasomes. Here, we investigated the role of Rip2, the adaptor for Nod1- and Nod2-dependent signalling, in multiple aspects of the host response to muramyl peptides in vivo, such as inflammatory cytokine secretion, activation and recruitment of macrophages and neutrophils to the site of injection, systemic activation of myeloid, T and B cells in the spleen, adjuvanticity and capacity to polarize the adaptive response to ovalbumin. Our results demonstrate that Rip2 was crucial for all the biological functions studied. We also identified CD11c\textsuperscript{int}CD11b\textsuperscript{+} inflammatory dendritic cells as a major myeloid cell population responding to Nod stimulation in vivo. Together, our results highlight the importance of Rip2 for Nod-dependent induction of innate and adaptive immunity.

3.2 Introduction

The innate immune system plays a crucial role in host defense against invading pathogens, and relies on pattern recognition molecules (PRMs), which detect conserved microbial- and danger-associated molecular patterns (MAMPs or DAMPs). The best studied PRMs are the Toll-like receptors (TLRs), which localize either at the cell surface or within endosomes. More recently, intracellular cytosolic PRMs, such as the nucleotide oligomerization domain (Nod)-like receptors (NLRs) and the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), have been identified.

Nod1 and Nod2 are NLR proteins that trigger nuclear factor-\kappa B (NF-\kappa B) signalling in response to bacterial peptidoglycan. Specifically, Nod1 recognizes muramyl peptides containing meso-DAP (diaminopimelic acid) found in the peptidoglycan of most gram-negative bacteria and certain gram-positive bacteria (Chamaillard et al., 2003; Girardin et al., 2003a), whereas Nod2 recognizes muramyl dipeptide (MDP) produced in all bacteria (Girardin et al., 2003b; Girardin et
al., 2003c). Upon peptidoglycan detection, Nod1 and Nod2 recruit and associate with the adaptor protein Rip2, triggering pro-inflammatory pathways such as NF-κB and the mitogen-activated protein (MAP) kinases p38, JNK and ERK (Inohara et al., 2000; Kobayashi et al., 2002). Importantly, mutations in Nod2 are associated with increased risk to develop Crohn’s disease (Hugot et al., 2001; Ogura et al., 2001), an inflammatory disorder that affects the gastrointestinal tract.

The role of Nod1 and Nod2 in innate immunity has been extensively studied. Various epithelial cell lines have been shown to produce antimicrobial peptides following Nod1 (Sugawara et al., 2006) and Nod2 (Uehara et al., 2007) stimulation in vitro. Nod1 and Nod2 play a key role in the induction of antimicrobial peptides following infection with *Helicobacter pylori*, and *Listeria monocytogenes* (Boughan et al., 2006; Kobayashi et al., 2005). Moreover, Nod1 and Nod2 induce the production of nitric oxide, a molecule that is known to be directly microbicidal (Magalhaes et al., 2005; Park et al., 2007b; Totemeyer et al., 2006). Activation of Nod1 and Nod2 by live bacteria triggers pro-inflammatory responses leading to the induction of cytokine and chemokines (Opitz et al., 2005; Opitz et al., 2006). Using Nod-deficient mice, several in vivo studies have demonstrated a key role for Nod1 and Nod2 in host defence against pathogens such as *H. pylori*, *L. monocytogenes*, and *Staphylococcus aureus* (Hruz et al., 2009; Kobayashi et al., 2005; Viala et al., 2004).

In addition to contributing to innate immunity, Nod1 and Nod2 have been implicated in the induction of adaptive immune responses. Over two decades ago, pioneer studies already demonstrated that MDP and peptides containing DAP modulate adaptive immune responses to antigens by acting as a potent adjuvants (Adam et al., 1974; Kotani et al., 1975). The adjuvant activity of MDP was later found to be mediated via Nod2, since potentiation of the IgG response to ovalbumin (OVA) by MDP was abrogated in Nod2-deficient mice (Kobayashi et al., 2005). Moreover, recent studies by our group have shown that in vivo stimulation with Nod1 or Nod2 ligands results in a Th2-dependent antigen-specific adaptive immunity (Fritz et al., 2006; Magalhaes et al., 2008), providing further evidence of the role of these NLR proteins in adaptive immunity.

Rip2 was originally identified as a protein implicated in NF-κB activation and apoptosis induction (Inohara et al., 1998; McCarthy et al., 1998; Thome et al., 1998). Biochemical analyses
further demonstrated that Rip2 acts as an adaptor protein downstream of Nod1 and Nod2 (Inohara et al., 1999; Inohara et al., 2000). Initial studies using Rip2-deficient mice suggested that Rip2 was implicated in the responses to TLR ligands, as well as to Nod1 and Nod2 over-expression (Chin et al., 2002; Kobayashi et al., 2002; Lu et al., 2005). However, these initial observations linking Rip2 to TLR signalling have been strongly refuted by the use of backcrossed Rip2−/− mice. It is now clear, as demonstrated Park et al. that Rip2 deficiency affects cellular signalling and cytokine responses triggered by Nod1 and Nod2 ligands, but not TLR ligands (Park et al., 2007a).

While the role of Rip2 in mediating Nod1- and Nod2-dependent inflammatory signalling is clear, the implication of this adaptor protein in adaptive immunity is poorly understood. The two initial reports on Rip2−/− mice mentioned above suggested that these mice had intrinsic defective Th1 immune responses and that Rip2 was required for optimal activation of NF-κB and T cell proliferation upon TCR stimulation (Chin et al., 2002; Kobayashi et al., 2002). Rip2 was shown to associate with Bcl10, a signaling molecule of the TCR-induced NF-κB pathway, after T cell stimulation (Rueflı-Brasse et al., 2004). The same group also reported a delayed allograft rejection of neonatal heart tissue in Rip2-deficient mice, suggesting impaired adaptive immunity in the absence of Rip2 (Rueflı-Brasse et al., 2004). More recently, using fully backcrossed animals, other reports failed to identify intrinsic defects of the adaptive immune system in Rip2-deficient mice (Hall et al., 2008; Nembrini et al., 2008). Consistent with these results, Rip2 was recently found to be dispensable for the induction of an effective type 1 immune response during Toxoplasma gondii infection (Shaw et al., 2009). Moreover, using a vascularized transplant model, Fairhead et al. also reported that Rip2-deficient mice were fully able to mount Th1-mediated alloresponses to reject vascularized allografts (Fairhead et al., 2008).

Two recent reports have also provided evidence that Nod signaling could occur in a Rip2-independent manner. First, Shaw et al. observed that Nod2, but not Rip2, was involved in host defense to intraperitoneal challenge with Toxoplasma gondii, through a mechanism involving CD4+ T cells (Shaw et al., 2009). Second, Travassos et al. demonstrated that Nod-dependent induction of bacterial autophagy was Rip2-independent, and required direct interaction between Nod proteins and the autophagy protein ATG16L1 (Travassos et al., 2010). In addition, several lines of evidence indicate that MDP is also detected by NLRP3 and NLRP1, resulting in Rip2-independent activation of caspase-1 inflammasomes (Hsu et al., 2008; Martinon et al., 2004).
Therefore, considering the debatable role of Rip2 in adaptive immunity and the emerging concept that Nod-dependent or muramyl peptides-dependent pathways could be Rip2-independent, we sought to investigate, using in vivo models, the role of Rip2 in innate and adaptive immunity to the Nod1- and Nod2-activating muramyl peptides FK156 and MDP, respectively. Our results establish that Rip2 is absolutely required for all aspects of the inflammatory, innate and adaptive immune functions of muramyl peptides that we analyzed.

3.3 Materials and Methods

Mice

All animal experiments were approved by the Animal Ethics Review Committee of the University of Toronto. C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Rip2-deficient mice were obtained from Dr. Flavell, Yale University school of Medicine, and have been backcrossed thirteen times to C57BL/6 background. The animals were submitted to sanitary control tests and used at the age of 6–8 weeks. All animal experiments were performed according to local guidelines.

Reagents

MDP was purchased from EMD Biosciences (San Diego, CA) and synthetic FK156 ($\text{D}$-lactyl-$\text{L}$-Ala-$\text{g}$-$\text{D}$-Glu-$\text{meso}$-DAP-Gly) was obtained from Fujisawa Inc. (Japan). Specificity and purity of MDP, and FK156 were ensured as previously described (Magalhaes et al., 2005). Ovalbumin was purchased from Worthington-Biochemical Corporation (Lakewood, NJ), and the purity was ensured as recently reported (Fritz et al., 2007). Alum was obtained from Thermo Scientific (Waltham, MA) and ultrapure LPS was purchased from InvivoGen (San Diego, CA). Beside Alum and LPS, all reagents were free of endotoxin as determined by the Limulus Amebocyte Lysate Test (BioWhittaker).

Cytokine and chemokines dosage

Enzyme-linked immunosorbent assay (ELISA) for KC, MCP-1, and IL-6 were carried out according to the manufacturer’s recommendations (Duoset R&D Systems, Minneapolis, MN).
FK156 and MDP injection. For analysis of cytokine and chemokine responses, C57BL/6 (WT) or RIP2\(^{-/-}\) mice (6–8 weeks old) were injected intraperitoneal (i.p.) with endotoxin-free 1X PBS as a control, MDP, FK156 (both at 50µg in 100 µl per mouse diluted with endotoxin-free 1X PBS) or LPS (1µg in 100µl per mouse). At indicated time points, blood was collected at the tail vein (100 µl per sample), and serum was isolated from blood after coagulation (2h at RT) and centrifugation (10000rpm, 5 minutes). Subsequently, serum cytokines/chemokines were measured by standard ELISA procedures.

**Cell activation and recruitment**

Mice were injected with PBS, FK156 or MDP at 50µg/100µl/mouse i.p. and 24h after injection cell from the PC and spleen were isolated for recruitment and activation. For the isolation of PC cells, 5 ml of PBS-EDTA (0.5mM) was injected in the PC and the content was removed to collect intraperitoneal cells. Spleens were cut in small pieces, digested first with collagenase D (1mg/ml) and DNase I (200µg/ml) in RPMI supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1x non-essential amino acids and 50 mM β-mercaptoethanol (all purchased from Invitrogen) and 10% fetal calf serum (Hyclone) for 30 minutes at 37ºC, and then passed through a 100µm cell strainer. To remove red blood cells (RBC), each spleen was incubated with 1ml of RBC buffer (Sigma) for 5 minutes to lyse RBC. Cells were then washed with PBS, and spun twice to remove residual RBC buffer. Isolated splenocytes were used for FACS analysis.

**Flow cytometry**

Flow cytometry was performed as described previously (Fritz et al., 2007; Magalhaes et al., 2005). Briefly, single cell suspension were resuspended in FACS buffer (1x PBS containing 0.5% FCS, 0.05% sodium azide) and incubated with Mouse BD Fc Block™ purified anti-mouse CD16/CD32 mAb 2.4G2 (BD Biosciences, Mississauga, ON,) to block unspecific staining. Subsequently, the cells were incubated with antibodies obtained from BD Biosciences: Fluorescein isothiocyanate (FITC)-labelled antibodies for CD19, CD11b, NK1.1, CD4; biotin-labeled antibodies specific for CD69, MHC class II; phycoerythrin-(PE)-labelled antibodies specific for CD8, F4/80, CD62L, CD44, CD40; PerCP-Cy5.5-labelled antibodies specific for CD69; allophycocyanin-(APC)-labelled antibodies specific for TCRβ, CD11c, CD86 and APC-Cy7 Gr1 and matching isotype controls were used during staining. After staining, cells were
washed twice with FACS buffer and analyzed by FACS (FACSCalibur™ flow cytometer, BD Biosciences). FlowJo™ software was used for the analysis of the results. Data were collected on approximately 100,000 cells.

**Induction and analysis of antigen-specific T- and B-cell response**

For B-cell Ag-specific response, mice were immunized in a prime-boost protocol at day 0 and day 21, intraperitonially with a mixture of OVA (50 µg/mouse) and adjuvant (MDP, FK156 (50µg/100µl/mouse) and Alum (1:1 ratio with OVA)) in a final volume of 100 µl sterile endotoxin-free PBS. Blood from tail veins was collected at 21 days after the prime and 14 days after the boost immunization (35 days after the prime), and sera of individual mice were analyzed. For T-cell-specific response, 10 days after immunizations, splenocytes of immunization groups were re-stimulated ex vivo, and ELISPOT analysis were performed.

**ELISPOT**

Multiscreen plates (S2EM004M99 IL-4 and IL-5; Millipore, MA, USA) were coated (100 µl/well) with capture antibodies (IL-4 [clone: 11B11] at 2 mg/ml; IL-5 [clone: TRFK5] at 2 mg/ml; all purchased from Becton Dickinson, Mississauga, ON) and the assay was carried out as described previously (Fritz et al., 2007; Magalhaes et al., 2008). Results are expressed as the number of IL-4-, or IL-5-producing cells per 10^6 splenocytes ± standard deviation of duplicates.

**Analysis of B-Cell Responses**

Ig isotypes were analyzed by sandwich ELISA comparing serially diluted serum samples with an assay-intrinsic isotype-specific standard (capture Ab: goat anti-mouse Ig (H+L) was purchased from SouthernBiotech; IgG1 standard (15H6) was obtained from SouthernBiotech and IgG2c was obtained from Bethyl Laboratories Inc. (Montgomery, TX). Finally, biotinylated detection Abs for IgG1 and IgG2c were purchased from SouthernBiotech. For analysis of Ag-specific Ig isotypes against OVA, plates were coated with 10 µg/ml OVA in PBS in 0.1 M NaHCO₃ (pH 9.5). ELISAs were performed by coating standard Ig capture antibodies or the OVA antigen to 96-well plates (MaxiSorp; Nalgene Nunc, Rochester, NY). After washing and incubation with the isotype-control Abs for the standard or the serially diluted serum samples, the detection was performed by sequential incubation with biotinylated secondary Abs, HRP-conjugated
streptavidin (R&D Systems), and TMB (Sigma-Aldrich). The reaction was stopped with 2 \( \text{NH}_2\text{SO}_4 \) (Sigma-Aldrich) and absorbance was read at 450 nm using a victor plate reader (Perkin Elmer).

**Statistical analysis**

The results are given as mean ± standard error of the mean (SEM) with the exception of ELISPOT. Statistical analysis was performed with Graphpad Prism 5 software using a Mann-Whitney test; \( p<0.05 \) was considered significant.

### 3.4 Results

*Rip2 is required for Nod1- and Nod2-induced inflammation in vivo*

We first demonstrated that Rip2-deficient (Rip2 KO) and WT mice displayed similar numbers and proportions of the major immune cells (macrophages, neutrophils, conventional dendritic cells (DCs), B cells, naive and activated T cells, natural killer and natural killer T cells) in the peritoneal cavity (PC) and spleen (Figure 3.1). To assess the contribution of Rip2 to Nod-mediated inflammation in vivo, cytokine and chemokine levels in the serum were measured by ELISA following a 2h, 6h or 24h i.p. injection of WT and Rip2-deficient mice with PBS, FK156, or MDP. Injection of the Nod1 agonist, FK156, into WT mice resulted in a transient release of proinflammatory mediators, KC and IL-6, in the bloodstream peaking at 2h, while returning to nearly steady-state levels 24h after injection as previously shown (Magalhaes et al., 2005) (Figure 3.2A). This response was Rip2-mediated, since these cytokines were not detected in the serum of Rip2 KO mice (Figure 3.2A). Similarly, the Nod2 agonist MDP induced KC and IL-6 release in the serum of WT mice 2 and 6h following injection (Figure 3.2B). To demonstrate there is no intrinsic defect of Rip2 KO mice in mounting inflammatory responses in vivo, we also injected LPS into both WT and Rip2 mice to stimulate the TLR4 pathway, and found no difference in the production of cytokines (Figure 3.2A and 3.2B). Together, these results demonstrate that the release of both pro-inflammatory and pro-Th2 chemokines following intra-peritoneal stimulation with Nod ligands was fully Rip2-dependent.
Figure 3.1. Comparison of cell populations of WT and Rip2−/− mice.

Macrophage and neutrophil population from the PC of WT and Rip2−/− mice were stained with (A) antibodies specific for F4/80 and Gr1 and were analyzed by flow cytometry. Splenocytes from WT and Rip2−/− mice were stained with antibodies specific for (B) conventional DC population MHCIi+CD11c+hi, (C) B cell population CD19+MHCIi+, (D) Naive T cell (CD62L+CD44−), activated T cell (CD62L−CD44+) and effector memory T cell (CD62L−CD44+), (E) NK (NK1.1+), NKT (NK1.1lowTCRβ+) and T cell (TCRβ+) and (F) CD4 and CD8 T cell population. The data are represented as the mean ± SEM of 3 independent experiments (n=4-6).
Figure 3.2. Rip2 is required for Nod1- and Nod2-induced inflammation in vivo.

C57BL/6 (WT) or Rip2<sup>−/−</sup> mice were injected i.p. with endotoxin-free PBS, FK156 (50µg/100µl/mouse), MDP (50µg/100µl/mouse) or LPS (1µg/100µl/mouse). At 2, 6 and 24h post-injection, (A) inflammatory cytokines (KC and IL-6) and (B) pro-Th2 mediators (MCP-1) were measured in the serum of those mice by ELISA. Data are represented as the means ± SEM of three independent experiments (n=8-10). Significant differences between groups are indicated.

* p<0.05 using a Mann-Whitney test.
Rip2 is required for macrophage activation and neutrophil recruitment in vivo upon Nod1 and Nod2 activation

It has been previously demonstrated that Nod1 activation in vivo leads to macrophage activation and neutrophil recruitment (Magalhaes et al., 2005; Masumoto et al., 2006). However, the implication of Rip2 in the process is not defined. To this end, WT and Rip2-deficient mice were injected i.p. with Nod1 and Nod2 agonists and the activation of macrophages and the recruitment of neutrophils were analyzed by flow cytometry 24h post-injection. We observed that the total numbers of macrophages in the peritoneal cavity did not change upon Nod ligand stimulation, and were comparable between WT and Rip2-deficient mice injected with FK156 and MDP (Figure 3.3A). However, MDP and FK156 both triggered the activation of WT but not Rip2 KO peritoneal macrophages, as observed by flow cytometry using the surface marker MHC class II, whose cell surface expression increases in activated macrophages (Figure 3.3B). Next, the numbers of neutrophils (Gr1+CD11b+) were measured in the PC (Figure 3.3C) and spleen (Figure 3.3D) of WT and Rip2-deficient mice following ligand stimulation. A significant amount of neutrophils were recruited to the PC and the spleen of WT mice but not Rip2 KO mice following stimulation with Nod ligands (Figure 3.3C and D). Therefore, our results demonstrate that Rip2 plays a crucial role in the activation of inflammatory cells triggered by Nod1 and Nod2 ligands in vivo.
Figure 3.3. Rip2 is required for macrophage activation and neutrophil recruitment upon Nod1 and Nod2 activation in vivo.

C57BL/6 (WT) or Rip2$^{-/-}$ mice were injected i.p. with endotoxin-free PBS, FK156 or MDP at 50μg/100μl/mouse. At 24h post-injection, cells from PC and spleen were isolated, counted, and analysed by flow cytometry. (A) Total cell numbers of macrophages (CD11b$^{+}$F4/80$^{+}$) present in the PC were evaluated in each condition. (B) Mean fluorescence intensity (MFI) of cell-surface expression of major histocompatibility complex II (MHC II) on macrophage population present in the PC. (C, D) Total cell numbers of neutrophils (CD11b$^{+}$Gr1$^{+}$) present in the PC (C) and in the spleen (D) were evaluated in each condition. Data are represented as the means ± SEM of two independent experiments (n=4-6). Significant differences between groups are indicated. * p<0.05 using a Mann-Whitney test.
Rip2 modulates the activation of CD11c\textsuperscript{int}CD11b\textsuperscript{+} dendritic cells in the spleen upon Nod1/2 activation

We next aimed to identify the role of Rip2 in the activation of splenic antigen-presenting cells (APCs) by i.p.-injected Nod1 and Nod2 ligands. Splenocytes from WT and Rip2-deficient mice injected with PBS, FK156 or MDP were isolated after 24h, and stained with antibodies to CD11c, CD8 and CD11b. Using flow cytometry, cells were gated to identify macrophages/neutrophils/monocytes (CD11b\textsuperscript{+}CD11c\textsuperscript{−} gate R1), inflammatory CD11c\textsuperscript{int}CD11b\textsuperscript{+} dendritic cells (gate R2) and conventional DCs, CD11c\textsuperscript{hi} (gate R3) (Figure 3.4A). The conventional DCs, CD11c\textsuperscript{hi}, were further classified as CD8\textsuperscript{+}DCs (gate R4) and CD8\textsuperscript{−} DCs (gate R5) (Figure 3.4A). We first noticed that i.p. injection of Nod1 and Nod2 ligands did not modify the numbers of CD11b\textsuperscript{+} CD11c\textsuperscript{−} Gr1\textsuperscript{−} macrophages (data not shown) and CD11c\textsuperscript{hi} cells (Figure 3.4B and C) in both WT and Rip2 KO mice. Interestingly, we observed in contrast that the numbers of CD11c\textsuperscript{int}CD11b\textsuperscript{+} DCs in the spleen of WT but not Rip2-deficient mice strongly increased following injection with FK156 (1.49% ± 0.20) and MDP (1.09% ± 0.10) compared to the PBS control (0.66% ± 0.16) (Figure 3.4B and C). This result is of particular interest, given the fact that CD11c\textsuperscript{int}CD11b\textsuperscript{+} DCs have been implicated in several aspects of inflammation and host defense following bacterial infection (see Discussion).

The cell-surface expression of the co-stimulatory molecules CD40 and CD86 is up-regulated on APCs following activation and maturation of these cells (Rydstrom and Wick, 2007; Sundquist and Wick, 2005). We observed that the R2-gated CD11c\textsuperscript{int}CD11b\textsuperscript{+} population of inflammatory DCs displayed up-regulated expression of CD40 and CD86 in WT mice following FK156 and MDP stimulation, whereas this response was absent in Rip2-deficient mice (Figure 3.4D–G). On the contrary, no significant change in the expression of CD40 and CD86 in conventional DCs (CD11c\textsuperscript{hi}CD8\textsuperscript{+}CD11b\textsuperscript{−} and CD11c\textsuperscript{hi}CD8\textsuperscript{−}CD11b\textsuperscript{+}) was observed in the spleen of WT or Rip2-deficient mice i.p. injected with Nod1 and Nod2 ligands (Figure 3.4D–G). Overall, we describe here for the first time that Nod1 and Nod2 ligands trigger the recruitment and the activation of CD11c\textsuperscript{int}CD11b\textsuperscript{+} inflammatory DCs in vivo and further demonstrate that this process is dependent on Rip2.
Figure 3.4. Rip2 modulates the frequency and the activation of CD11c\(^{\text{int}}\)CD11b\(^{+}\) cells in the spleen upon Nod1 and Nod2 activation.

C57BL/6 (WT) or Rip2\(^{-/-}\) mice were injected i.p. with endotoxin-free PBS, FK156 or MDP at 50\(\mu\)g/100\(\mu\)l/mouse. 24h post-injection spleen cells were stained with Abs to CD11c, CD8 and CD11b. (A) Representative dot plots from WT splenocytes are shown here with different gatings. The R1 gate (CD11b\(^{+}\)CD11c\(^{-}\)) represents monocytes/macrophages and neutrophils, the R2 gate contains the inflammatory CD11c\(^{\text{int}}\)CD11b\(^{+}\) DCs and the R3 gate (CD11c\(^{\text{hi}}\)) shows conventional DCs. The conventional DC subsets were further identified as gate R4 CD11c\(^{\text{hi}}\)CD11b\(^{-}\)CD8\(^{+}\) (CD8\(^{+}\) DCs) and gate R5 CD11c\(^{\text{hi}}\)CD11b\(^{+}\)CD8\(^{-}\) (CD8\(^{-}\) DCs containing CD4\(^{+}\) and CD4\(^{-}\) populations). (B) The frequencies of inflammatory CD11c\(^{\text{int}}\)CD11b\(^{+}\) DCs are shown in the dot plots at 24h post-injection. (C) The absolute number of R2, R4 and R5 populations in the spleen of C57BL/6 and Rip2\(^{-/-}\) mice at 24h post-injection. (D and F) Expression of CD40 and CD86 co-stimulatory molecules on R2, R4 and R5 populations isolated from the spleen of C57BL/6 or Rip2\(^{-/-}\) mice at 24h after i.p. injection of endotoxin-free PBS, FK156 or MDP at 50\(\mu\)g/100\(\mu\)l/mouse. (E and G) MFI of cell-surface expression of CD40 and CD86 on R2, R4 and R5 populations. Isotype controls are shown in (D–G). For all panels, data are represented as the means ± SEM of three to four independent experiments (n=8–10). Significant differences between groups are indicated. * \(p<0.05\) using a Mann-Whitney test.
FK156 has been shown to have a costimulatory signal after TCR engagement by increasing the capacity of T cells to proliferate following concanavalin A (ConA) stimulation in vitro (Weston et al., 1991). Another report described similar results by showing that intestinal T cells, restimulated in vitro with phytohaemagglutinin or ConA, displayed increased proliferation in mice that had received MDP intragastrically compared with vehicle control (Zunic et al., 1996). Here, we examined whether i.p. injection of FK156 or MDP could activate splenic T cells in vivo, using the CD69 activation marker and whether this process was dependent on Rip2. Flow cytometry data showed a significant upregulation of CD69 cell-surface expression on WT splenic T cells following i.p. injection with FK156, and this response was abrogated in Rip2-deficient mice (Figure 3.5A). Moreover, we further showed that FK156 injection resulted in the activation of both CD4+ and CD8+ splenic T cells, thus showing that the activation was not restricted to a particular type of T cells (Figure 3.6). On the contrary, i.p.-injected MDP failed to stimulate splenic T cells in vivo (Figures 3.5A and 3.6). Interestingly, FK156 and MDP induced strong activation of CD19+CD69+ B cells in the spleen of WT but not Rip2-deficient mice (Figure 3.5B). In addition, we observed a significant increase in B-cell numbers in the spleen of WT mice but not of Rip2-deficient mice injected with FK156 and MDP, whereas T-cell numbers appeared to remain constant (Figure 3.5C). In conclusion, our results demonstrate that Nod1 and Nod2 ligands trigger a systemic response that results in splenic lymphocyte recruitment and activation, and that these responses are dependent on Rip2.
Figure 3.5. Rip2 modulates the activation of T and B cells in the spleen upon Nod1 and Nod2 activation.

C57BL/6 (WT) and Rip2−/− mice were injected i.p. with FK156, MDP, or PBS as a control. After 24h, splenocytes were isolated, stained with markers for T cell (TCRβ) and B cell (CD19) and analyzed by flow cytometry. The frequencies of activated (A) T cell (TCRβ+CD62L+CD69+) and (B) B cell (CD19+CD69+) are shown in the dot plots at 24h post-injection. (C) The absolute number of activated T- and B-cell populations in the spleen of C57BL/6 and Rip2−/− mice at 24h post-injection. For all panels, data shown are represented as the means ± SEM of three independent experiments (n=6-8). Significant differences between groups are indicated. * p<0.05 using a Mann-Whitney test.
Figure 3.6. Rip2 modulates the activation of CD4\(^+\) and CD8\(^+\) T cells in the spleen upon Nod1 and Nod2 activation.

C57BL/6 (WT) and Rip2\(^{-/-}\) mice were injected i.p. with FK156, MDP, or PBS as a control. After 24h, splenocytes were isolated and stained with markers for CD4 T cell (TCR\(\beta^+\)CD4\(^+\)) and CD8 T cell (TCR\(\beta^+\)CD8\(^+\)) and analysis by flow cytometry. The frequencies of activated (A) CD4 T cell (TCR\(\beta^+\)CD4\(^+\)CD69\(^+\)) and (B) CD8 T cell (TCR\(\beta^+\)CD8\(^+\)CD69\(^+\)) are shown in the dot plots at 24h post-injection. The data are represented as the mean ± SEM of two independent experiments (n=4–5). The asterisk indicates statistically significant differences (p<0.05) using a Mann-Whitney test.
Rip2 is essential for Nod1- and Nod2-induced Th2 immunity

Our recent findings show that Nod1 and Nod2 activation drives a Th2-biased immune response (Fritz et al., 2007; Magalhaes et al., 2008). To determine if Rip2 was required for the induction of antigen (Ag)-specific Th2 immune response upon Nod1 and Nod2 activation, we immunized WT and Rip2-deficient mice i.p. with OVA plus FK156 or MDP, used as a adjuvant. Compared with the effect of immunizations with OVA only, WT mice given a mixture of OVA plus FK156 or OVA plus MDP elicited markedly higher numbers of IL-4- and IL-5-producing splenocytes upon restimulation with OVA protein (Figure 3.7A), confirming our previous results (Fritz et al., 2007; Magalhaes et al., 2008). Importantly, the Th2 response elicited upon coinjection of OVA plus FK156 or OVA plus MDP was strictly Rip2-dependent, as no significant production of Ag-specific IL-4- and IL-5-producing cells over background levels could be detected in Rip2-deficient mice (Figure 3.7A). Moreover, analysis of the B-cell population revealed that in WT mice, OVA-specific Ig, IgG1, was predominantly induced after immunizations with FK156 or MDP, whereas the induction of IgG1 was abrogated in Rip2-deficient animals (Figure 3.7B). As a control, both WT and Rip2-deficient mice were injected with OVA or OVA plus aluminum hydroxide (Alum), a strong Th2-inducing adjuvant, which showed equivalent IgG1 production in these mice (Figure 3.7C). Our results therefore show that FK156 and MDP elicit priming of Th cell immune responses with a Th2 polarization profile and subsequent IgG1 production in a Rip2-dependent manner. In summary, we demonstrated that Rip2 is required and critical for Nod1- and Nod2-driven Th2 immunity in vivo.
Figure 3.7. Rip2 is essential for Nod1- and Nod2-induced Th2 immunity.

(A) WT or Rip2−/− mouse were injected i.p. with OVA, OVA+FK156 or OVA+MDP. Ten days after immunization, splenocytes from immunized animals were restimulated ex vivo with medium or OVA. The frequencies of OVA-specific producing IL-4 or IL-5 per million splenocytes were determined by ELISPOT analysis. A prime-boost immunizations (day 0 and day 21, respectively) protocol was carried out on WT or Rip2−/− injected i.p. with (B) OVA, OVA+FK156 or OVA+MDP and (C) OVA or OVA+Alum. Serum of each animal was taken on day 21 (prime) and day 35 (boost), and OVA-specific IgG1 was analyzed by ELISA. Data shown are representative of three independent experiments for both (A) and (B). The bar represents the means ± SEM of each cohort (n=2–3 for (A), n=3–4 for (B) and n=2–3 for (C)). The asterisk indicates statistically significant differences (p<0.05) using a Mann-Whitney test.
3.5 Discussion

In this study, we aimed to analyze in detail the contribution of Rip2 to the immune responses triggered by the Nod ligands FK156 and MDP in vivo. To this end, we investigated in WT and Rip2-deficient mice a number of host responses to these bacterial molecules: inflammatory cytokine secretion; activation and recruitment of macrophages and neutrophils to the site of injection; systemic activation of APCs, T and B cells in the spleen; adjuvanticity; and capacity to polarize the adaptive response to OVA. Strikingly, we demonstrated that all aspects of the immune response to FK156 and MDP studied here were fully Rip2-dependent. In addition, our results also identified CD11c<sup>int</sup>CD11b<sup>+</sup> inflammatory monocytes as a major myeloid cell population responding to Nod stimulation in vivo, again in a fully Rip2-dependent manner.

Rip2 is an adaptor protein of Nod1- and Nod2-dependent signalling, which was initially shown by biochemical analyses to bridge Nod proteins to the IκB kinase (IKK) complex, leading to NF-κB activation (Inohara et al., 2000). Recent results in Rip2-deficient mice further established that Rip2 played a critical role not only in NF-κB, but also in mitogen-activated protein kinase signalling, in macrophages stimulated with Nod ligands, which correlated with an absolute requirement for Rip2 in mediating inflammatory cytokine secretion following in vivo injection of Nod1 ligands (Kobayashi et al., 2002). However, recent evidence suggests that Nod-dependent but Rip2-independent signalling also exists. Indeed, Nod-dependent induction of autophagy was shown to occur in Rip2-deficient cells, and this involves the direct recruitment of the autophagy protein ATG16L1 to the activated Nod complex (Travassos et al., 2010). Moreover, host response to T. gondii infection requires Nod2 and not Rip2, and this seems to be associated with the induction of Nod2-dependent and Rip2-independent pathway in the CD4<sup>+</sup> T cells of infected mice (Shaw et al., 2009). Therefore, and because autophagy and T-cell signalling are both crucial elements of the immune response, we aimed to identify if specific aspects of the host response to the muramyl peptides FK156 and MDP could occur in Rip2-deficient mice. Our data establish that Rip2-independent pathways, including the ones discussed above, do not significantly contribute to the modulation of immune responses triggered by the Nod ligands FK156 and MDP.

The host NLR proteins Nod1 and Nod2 play key roles in the host response to muramyl peptides. However, several lines of evidence suggest that MDP can also trigger NLRP3- and NLRP1-
dependent activation of caspase-1 inflammasomes, resulting in the induction of IL-1β and IL-18 secretion (Hsu et al., 2008; Martinon et al., 2004). Importantly, these two cytokines control numerous aspects of innate and adaptive immunity, and inflammasome induction is believed to occur in a Rip2-independent manner. Therefore, an interesting possibility is that muramyl peptide-triggered inflammasome signalling could still occur in Rip2 KO mice, and participate in the host immune response to these bacterial molecules. In agreement with this idea is the fact that full MDP-induced IL-1β secretion has been shown to require NLRP3 (Martinon et al., 2004). Our data, however, failed to identify Rip2-independent roles for the muramyl peptides FK156 and MDP in immune regulation. This suggests that MDP-triggered NLRP3 or NLRP1 signalling is not, in itself, sufficient to significantly contribute to innate or adaptive immune responses to this muramyl peptide. It remains possible, however, that both Rip2- and NLRP-dependent signalling could cooperate to generate a full-blown host immune response to muramyl peptides. This interesting possibility could not be tested in Rip2 KO mice only, and awaits further studies in NLRP3- and NLRP1-deficient mice.

An unexpected result from our studies was the discovery of a specific cell population of the myeloid lineage, the CD11c^{int}CD11b^{+} inflammatory cells, that was recruited and activated in the spleen of animals i.p. injected with Nod1 and Nod2 ligands, whereas macrophages and conventional DCs populations were not. Previous studies have identified that these cells have phenotypic characteristics of both macrophages and DCs, that they are recruited and activated in inflammatory conditions in both draining lymph nodes and the spleen, and play a role in host defense against bacterial pathogens, including Salmonella (Sundquist and Wick, 2005, 2009). Interestingly, these CD11c^{int}CD11b^{+} inflammatory cells, also known as inflammatory monocytes, are likely to be equivalent to the TNF/iNOS-producing DCs (Tip-DCs), originally described by the group of Pamer (Serbina et al., 2003). Indeed, while studying the host response to systemic Listeria infection, Serbina et al. observed that Listeria infection triggered the CCR2-dependent recruitment and activation of TNF- and iNOS-producing myeloid cells into the spleen of infected animals. These cells were the major source of TNF and iNOS in the spleen following infection, and therefore were critical for host resistance to this bacterial pathogen. Interestingly, the authors also demonstrated that the recruitment of Tip-DCs to the spleen occurred in a MyD88-independent manner, but required secretion of MCP-1 and the expression of the chemokine receptor CCR2. Moreover, they showed that these events required the cytosolic
escape of the bacterium, thus suggesting that cytosolic PRMs likely contribute to their recruitment into the spleen. Therefore, our results fully support these original observations, by showing that the intracellular PRM proteins Nod1 and Nod2 trigger MCP-1 secretion and play a key role in the splenic recruitment and activation of cells that share phenotypic characteristics with Tip-DCs. Further study is required to demonstrate that the CD11c\textsuperscript{int}CD11b\textsuperscript{+} inflammatory cells identified here in the spleen of mice injected with Nod ligands indeed produce TNF and iNOS, and that they are recruited in a CCR2-dependent manner.

In summary, our work highlights the critical role played by the adaptor molecule Rip2 in all aspects of the immune response to Nod1 and Nod2 ligands. These observations imply that recently identified Nod- or muramyl peptide-triggered Rip2-independent pathways do not significantly contribute, on their own, to the immune response to these bacterial molecules. Further study is needed to decipher how Nod1 and Nod2, which both rely extensively on the same downstream adaptor protein, can nonetheless trigger nonoverlapping functions, as illustrated, for instance, by the implication of Nod2 mutations in Crohn’s disease.
Chapter 4
pH-dependent Internalization of Muramyl Peptides from Early Endosomes Enables Nod1 and Nod2 Signaling

This chapter was published as


Contribution of Data

All experiments/analyses were performed by Jooeun Lee, unless noted otherwise;

Figure 4.6, panel A – *SLC15* expression analysis by RT-PCR was provided by Ivan Tattoli.

Figure 4.8 was obtained from Kacper A. Wojtal as part of a larger study, which was reported elsewhere (Wojtal et al., 2009)
4

4.1 Abstract

Nod1 and Nod2 are members of the Nod-like receptor family that detect intracellular bacterial peptidoglycan-derived muramyl peptides. The biological effects of muramyl peptides have been described for over three decades, but the mechanism underlying their internalization to the cytosol remains unclear. Using the human epithelial cell line HEK293T as a model system, we demonstrate here that Nod1-activating ligands entered cells through endocytosis, most likely by the clathrin-coated pit pathway, as internalization was dynamin-dependent but not inhibited by methyl-β-cyclodextrin. In the endocytic pathway, the cytosolic internalization of Nod1 ligands was pH-dependent, occurred prior to the acidification mediated by the vacuolar ATPase and was optimal at pH ranging from 5.5 to 6. Similarly, the Nod2 ligand MDP was internalized into host cytosol through a similar pathway with optimal pH for internalization ranging from 5.5 to 6.5. Moreover, Nod1-activating muramyl peptides likely required processing by endosomal enzymes, prior to transport into the cytosol, suggesting the existence of a sterically gated endosomal transporter for Nod1 ligands. In support for this, we identified a role for SLC15A4, an oligopeptide transporter expressed in early endosomes, in Nod1-dependent NF-κB signaling. Interestingly, SLC15A4 expression was also up-regulated in colonic biopsies from patients with inflammatory bowel disease, a disorder associated with mutations in Nod1 and Nod2. Together, our results shed light onto the mechanisms by which muramyl peptides get access to the host cytosol, where they are detected by Nod1 and Nod2, and might have implications for the understanding of human diseases, such as inflammatory bowel disease.

4.2 Introduction

Innate immunity relies on the detection of conserved microbial- or danger-associated molecular patterns (MAMPs or DAMPs), by pattern-recognition molecules. In mammals, several families of pattern-recognition molecules have been recently identified, including the transmembrane Toll-like receptors (TLRs), cytosolic Nod-like receptors (NLRs) and RIG-I-like receptors (RLRs) (Akira et al., 2006). NLR proteins include Nod1 and Nod2, which trigger pro-inflammatory pathways such as NF-κB and mitogen-activated protein kinases, in response to bacterial peptidoglycan (Fritz et al., 2006), and NLRPs (also known as Nalps), such as NLRP1
and NLRP3, which induce the activation of caspase-1 inflammasomes in response to various MAMPs and DAMPs (Benko et al., 2008).

In the case of TLRs, there is accumulating evidence that the subcellular localization and the function of these pattern-recognition molecules is tightly associated, at multiple levels, with endocytosis and phagocytosis, which represent evolutionary conserved mechanisms for the internalization of small (<0.5µm) and large (>0.5µm) particles, respectively. Indeed, whereas some TLRs are expressed at the plasma membrane, others (such as TLR3, -7 and -9) are found predominantly associated with the endoplasmic reticulum and endosomal compartments, where they detect their respective microbial-derived nucleic acid MAMPs (Akira and Takeda, 2004). In particular, TLR9 has been shown to move from the endoplasmic reticulum to CpG DNA-containing endosomes, concurrent with the accumulation of MyD88, thus showing that endosomes represent the physiological location where TLR9-dependent signaling arises (Latz et al., 2004). In addition, studies on TLR4 have demonstrated that lipopolysaccharide (LPS) is endocytosed by a receptor-mediated mechanism dependent on dynamin and clathrin and co-localized with TLR4 on early/sorting endosomes (Husebye et al., 2006). In the case of this TLR, it is believed that endosomal trafficking is associated with termination of the MyD88-dependent pro-inflammatory signal (Husebye et al., 2006). In contrast, TLR4 in early endosomes has been shown recently to engage TRAM and TRIF adaptors, resulting in the ignition of type I interferon signaling in response to LPS (Kagan et al., 2008). Therefore, the nature of the cellular response to LPS is dependent upon the subcellular localization of TLR4, thus reinforcing the importance of the interplay between TLR signaling and endosomal trafficking.

A number of studies have also linked TLR signaling with phagosome maturation. While it remains controversial whether TLR-dependent signaling actually drives phagosomal maturation (Blander and Medzhitov, 2004; Yates and Russell, 2005), it is clear that the processing of engulfed microbes within phagosomes regulates the availability of MAMPs within this compartment. Accordingly, Herskovits et al. have recently demonstrated that, in interferon γ-activated macrophages, the degradation of *Listeria monocytogenes* in the phagolysosome generates bacterial molecules, which could specifically trigger type I interferon responses through a Nod2-dependent pathway (Herskovits et al., 2007). This interesting observation suggests that innate immune signaling and microbial degradation within the phagolysosome are
processes that are intimately linked. It also provides support to the concept that Nod-dependent signaling is associated with intracellular vesicular trafficking.

Nod1 and Nod2 both detect specific structures from bacterial peptidoglycan (Girardin et al., 2003c). While Nod2 detects muramyl dipeptide (MDP) (Girardin et al., 2003b; Inohara et al., 2003), a motif found in almost all bacteria, Nod1 specifically senses diaminopimelic acid (DAP)-containing muramyl peptides (Chamaillard et al., 2003; Girardin et al., 2003a). In particular, human Nod1 preferentially detects N-acetylmuramyl-L-Ala-D-Glu-mesoDAP (M-Tri-DAP) (Magalhaes et al., 2005), and the minimal motif for Nod1-dependent sensing is the dipeptide D-Glu-mesoDAP (iE-DAP) (Chamaillard et al., 2003; Girardin et al., 2003c). Interestingly, long before the identification of Nod1 and Nod2 as sensors of muramyl peptides and bacterial peptidoglycan, the biological activities of these bacterial-derived molecules had been studied extensively (Lederer, 1980, 1986). It is well documented that these muramyl peptides trigger a multitude of immune responses, such as the induction of cytokines/chemokines, the production of nitric oxide and reactive oxygen species and the clearance of microbes by phagocytic cells (Lederer, 1980, 1986). A considerable literature also demonstrated that these muramyl peptides synergize with MAMPs detected by TLRs, such as LPS (Traub et al., 2004). Although the identification of Nod1 and Nod2 as sensors of muramyl peptides has provided an acceleration in this field of investigation, it also brought the question of how such microbial molecules could get access to the host cytosol, where Nod1 and Nod2 reside. Interestingly, research aiming at improving the biological activities of these muramyl peptides demonstrated early on that the addition of lipophilic groups to these molecules enhanced their activity considerably, suggesting that their internalization was likely a key factor in determining their efficiency (Fogler and Fidler, 1987; Mehta et al., 1982; Nayar et al., 1986; Schroit and Fidler, 1982).

The mechanisms by which muramyl peptides get access to the host cytosol remain unclear. This question is of fundamental importance for our understanding of Nod-dependent signaling, and potentially holds broad therapeutic implications. Indeed, mutations in Nod1 and Nod2 have been associated with inflammatory bowel disease (IBD) in humans (Le Bourhis et al., 2007). In particular, Nod2 has been identified as the first susceptibility gene for Crohn’s disease (Hugot et al., 2001; Ogura et al., 2001).
In this report, we used the HEK293T epithelial cell line to study the mechanism of internalization of Nod1 ligands. We demonstrated that these peptidoglycan-derived molecules enter cells by endocytosis, and that the composition of the Nod1-activating molecules dramatically affected their intrinsic uptake capacity. Our data also suggested that this internalization was mediated by clathrin-dependent endocytosis, as internalization of Nod1 ligands required dynamin and was independent from caveolae. Further, we showed that, within endosomes, the internalization of Nod1 ligands was critically dependent on pH, and was optimal at pH ranging from 5.5 to 6, which are characteristic of early endosomes. Accordingly, internalization of Nod1-activating molecules did not require the action of the vacuolar ATPase (V-ATPase) complex. We also provide evidence that the Nod2 ligand MDP enters cells through a similar endocytic process. Our results also show that the internalization of Nod1 ligands is a process that is sterically gated, and likely requires the action of hydrolytic endosomal enzymes prior to transport into the cytosol, thus suggesting the existence of one or several specific transporters for Nod1 ligands in early endosomes. Using knockdown assays, we identified SLC15A4 as a putative transporter for Nod1 ligands in early endosomes. SLC15A4 expression was found to be significantly upregulated in tissue biopsies from IBD patients, therefore highlighting a potential role for the modulation of peptidoglycan access to the cytosol in IBD etiology. Together, our results uncover the mechanism by which Nod ligands traffic into cells and get access to the cytosol where they are detected by Nod1 and Nod2. Our observations also highlight the previously unappreciated link between endosomal acidification/maturation and Nod-dependent signaling.

4.3 Materials and Methods

Cell culture and reagents

Human embryonic kidney epithelial cell line HEK293T (American Type Culture Collection) was cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 5% fetal calf serum and 1% penicillin/streptomycin. Cells were maintained in 95% air, 5% CO2 at 37°C. Endotoxin-free fetal calf serum and phosphate buffer saline were from Wisent (St-Bruno, Quebec). Fetal calf serum was used after heat inactivation at 56°C for 30 min. All cell culture reagents and antibiotics were also from Wisent.
Nod1 ligands, iE-DAP (γ-D-Glu-mesoDAP), Tri-DAP (L-Ala-D-Glu-mesoDAP), M-Tri-DAP (MurNAc-L-Ala-D-Glu-mesoDAP), C12-iE-DAP, were from InvivoGen. iE-DAP, Tri-DAP and M-Tri-DAP were used at concentrations ranging from 1 to 10 μg/ml whereas C12-iE-DAP was used from 0.01 to 1 μg/ml. Nod2 ligand, MDP was also from InvivoGen and used at a concentration of 10 μg/ml.

The inhibitors of endocytosis were purchased from Sigma and used at the following concentrations: valinomycin (1-10 μM), bafilomycin A1 (10-50 nM), dynasore monohydrate (80 μM), nocodazole (1 μg/ml), cytochalasin D (1 μg/ml), carbonyl cyanide m-chlorophenylhydrazone (CCCP) (1-10 μM), methyl-β-cyclodextrin (2.5 to 10 mM).

**Extracellular acidification assays**

To examine the effect of extracellular pH on ligand uptake, isotonic buffer (IB) (50 mM HEPES, pH 7, 100 mM KCl, 3 mM MgCl₂, 0.1 mM dithiothreitol, 85 mM sucrose, 0.2% bovine serum albumin, 1 mM ATP and 0.1 mM GTP) was prepared with pH modified to 5, 5.5, 6, 6.5, or 7, and ATP, dithiothreitol and GTP were added freshly for each experiment. Cells were then incubated for 1 hour at 37°C with the respective ligands in 500 μl IB at varying pH. Subsequently, IB was removed and replaced with DMEM plus 5% fetal-calf serum for 5 h before performing luciferase measurements.

**NF-κB activation assays**

Transfections were carried out using polyethylenimine (Polysciences Inc., Warrington, PA) in HEK293T according to the manufacturer’s instructions. Briefly, cells were transfected overnight with 75 ng of NF-κB luciferase reporter plamid (Igκ-luc, Invitrogen). The empty vector (pcDNA3.1, Invitrogen) was used to balance the transfected DNA concentration. The expression vector for human Nod2 (0.2 ng/well) was a kind gift from Dr. Nunez (University of Michigan, Ann Arbor, MI). Following transfection, Nod1 or Nod2 ligands were added the next day for 6 hours (unless specified) before performing luciferase measurements. For NF-κB activation assays in digitonin-permeabilized cells, HEK293T were incubated for 10 min at 37°C with Nod1 ligands in isotonic digitonin buffer (DB) (500 μl) with or without 10 μg/ml digitonin (Sigma), and then placed in DMEM for 6 h, as previously described (Girardin et al., 2003a).
As a control for the possible toxicity of the trafficking inhibitors used towards NF-κB activation, we systematically transfected over-night the Igκ-luci reporter vector together with 50 ng of expression vector encoding for Rip2 (from Dr Thome, UNIL, Lausanne), the Nod1 adaptor protein acting downstream of the detection of peptidoglycan by Nod1. In all the experiments presented in this study, the doses of the drugs used had no effect on Rip2-mediated activation of NF-κB (data not shown). NF-κB-dependent luciferase assays were performed in duplicate, and data represent at least three independent experiments. Data show mean ± S.E.

**Immunofluorescence**

HEK293T cells were incubated in DMEM without fetal calf serum for 1 h prior to incubation in IB at pH 5.5 or 7 plus transferrin conjugated to Alexa Fluor 568 (10 µg/ml, Molecular Probes Inc.) for 1 h at 37°C. Rab5-RFP expression vector was a kind gift from Dr. John Brumell (the Hospital for Sick Children, Toronto). SLC15A4-V5 was obtained from Dr. Knipp (The State University of New Jersey). Cells were co-transfected with either Rab5-RFP (500 ng) or SLC15A4-V5 (500 ng) or both overnight, and incubated in IB at pH 5.5 or 7 for 1 h at 37°C the next day. Cells were then washed with cold phosphate-buffered saline, fixed for 15 min at room temperature with paraformaldehyde (3.7% w/v in phosphate-buffered saline), permeabilized with 0.1% TritonX-100 for 5 min, stained with anti-V5 (anti-mouse-fluorescein isothiocyanate) for 1 h and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Coverslips were visualized on a Carl Zeiss Axiovert 200M microscope with a 63X oil fluorescence objective.

**Semi-quantatative PCR**

Total RNA samples from untreated cells were prepared according to the manufacturer’s protocols (Qiagen). After genomic DNA elimination with RNase-free DNase, the complementary DNA was generated from 1 µg RNA using a mixture of oligo(dT) and random primers and Omniscript Reverse Transcriptase (Qiagen). PCR was performed with *Taq* polymerase (Invitrogen) using the following primers: 5’-TTGCTTCTGCTTGCTGCTG3’, 5’-GCCCCTGACATGAAATATGG 3’ for SLC15A1, 5’-GCCCTGCTTGCAAGCGTTTTT- 3’, 5’-AGAGTCTCTGGGGCCTTGT-3’ for SLC15A2, 5’-GCTTAAGCTCGCTCTCCAAA-3’,5’-GCAAGATCTTCAACCACACGAC-3’ for SLC15A3, 5’-AGCGATCCCTGCTGTTAGGTCG-3’, 5’-AGGAGGCTTGGTGTAGAAAA-3’ for SLC15A4.
**Design and construction of siRNA sequences against human SLC15A4**

The sequences of the siRNA specifically targeting SLC15A4 gene were purchased from Ambion (Silencer Pre-designed siRNA) siRNA ID # S42440 and siRNA ID #S42441 or designed through siRNA Wizard v3.0 (InvivoGen). The sequences were the following: 5’ CGGCTGCTATTTGAACTAT 3’ for #B, 5’GCAGACAACATATGTTTTA 3’ for #A, 5’GAGTCTTTTCAGCAATCTTCTA 3’ for #C, 5’ GATTCATGTAAGATGTCTCAT 3’ for #D 5’GTGGAGAGCGCCAGAGTAA 3’ for #E, 5’ GATTCATGTAAGATGTCTCAT 3’ for #F, and 5’ CGGCTGCTATTTGAACTAT 3’ for scrambled. Two different vectors were used to generate shRNA against SLC15A4; pLKO.1 vector (Addgene, Cambridge, MA) for lentiviral knockdown system, and psiRNA-h7SK-GFPzeo transient knockdown of SLC15A4 expression. For both vectors, positive clones were identified by restriction digestion and confirmed by sequencing.

**Lentiviral vector cloning**

The sense and anti-sense oligonucleotides were resuspended in water at 20 μM, and annealed by incubating at 95°C for 4 min, 70°C for 10 min, then decreasing the temperature to room temperature slowly (0.1°C/min). The resulting lentiviral shRNA vector was confirmed by restriction enzyme digestion with Agel/EcoRI. The constructed vector was also confirmed by DNA sequencing with pLKO.1 sequencing primer (5’- CAA GGC TGT TAG AGA GAT AAT TGG A-3’).

**Lentivirus packaging**

Packaging, and purification of the lentivirus were performed according to classical procedures. Briefly, HEK293T cells were seeded 1.5 x 10^6 in a 10-cm culture dish, in DMEM supplemented with 10% fetal bovine serum without antibiotics. The following day, cells were co-transfected with the lentiviral vector (1 μg), and the lentiviral packaging/envelope vectors psPAX2 (750 ng), pMD2.G (250 ng). Viruses were collected from the culture supernatant 48h post-transfection, spun at 1250 rpm for 5 min, and the media passed through a 0.45-μm filter.

**Lentiviral transduction in HEK293T cells**
Cells were seeded in either 6-well plates or 24-well plates, and then incubated with lentivirus for 3 days. Polybrene (10 µg/ml) was added to increase the efficiency of lentiviral transduction. Following transduction, cells were co-transfected with NF-κB luciferase constructs and luciferase assays were performed as above.

*Western blotting*

HEK293T cells were centrifuged, and the pellet was lysed with radioimmune precipitation assay buffer (0.5M Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, protease inhibitor mixture (Sigma P8340)). Total cell lysates were boiled for 10 min, and subsequently subjected to SDS-PAGE. Western blot was performed according to a standard protocol, using a rabbit polyclonal anti-SLC15A4 (Santa Cruz Biotechnology, dilution 1:500), followed by incubation with a secondary anti-rabbit horseradish peroxidise antibody (Thermo Scientific, 1:10,000) and an enhanced Chemiluminescence Kit (PerkinElmer, Life Sciences). Mouse monoclonal anti-tubulin (Sigma, T9026, 1:10,000) was used as loading control.

*SLC15A4 expression in IBD patients*

The analysis of SLC15A4 expression in 49 CD patients and 53 UC patients was performed in the context of a larger study, which was reported elsewhere (Wojtal et al., 2009).

### 4.4 Results

HEK293T cells transfected overnight with an expression vector encoding for luciferase under the control of an NF-κB-responsive promoter (Igκ-luci) were stimulated with increasing concentrations of the Nod1 ligands iE-DAP, Tri-DAP or M-Tri-DAP for 6 h. iE-DAP represents the minimal peptide activating Nod1, whereas Tri-DAP is the minimal naturally occurring Nod1 ligand, and M-Tri-DAP is the minimal muramyl peptide stimulating Nod1 (Chamaillard et al., 2003; Girardin et al., 2003c) (Figure 4.1A). Although Tri-DAP and M-Tri-DAP activated cells comparably, iE-DAP appeared to be a very poor agonist, even at the highest dose tested (10 µg/ml) (Figure 4.1B). We next stimulated cells for 1–6 h with 10 µg/ml of Tri-DAP or M-Tri-DAP, and observed that these Nod1 ligands required at least 3–4 h of stimulation to induce measurable activation of the NF-κB reporter gene (Figure 4.1C). We and others have previously
demonstrated that iE-DAP was able to trigger Nod1-dependent NF-κB activation in a setting where the ligands were added overnight together with a liposomal transfection reagent (Chamaillard et al., 2003; Girardin et al., 2003c). Therefore, the results above likely reflect the fact that iE-DAP, contrary to Tri-DAP or M-Tri-DAP, is inefficiently internalized by HEK293T cells. In order to study this question further, we used a derivative iE-DAP that contains a lauroyl (C12) group attached to the glutamic residue of iE-DAP (see Figure 4.1A). The rationale for such a modification is that the fatty acids may form micelle-like structures and display enhanced internalization. We observed that the addition of the fatty acid moiety to iE-DAP enhanced >100 fold the capacity of the Nod1 ligand to trigger NF-κB (Figure 4.1D). This suggests that iE-DAP activated cells poorly as a result of defective trafficking, rather than because of an intrinsic inefficiency to stimulate Nod1. Next, we questioned if the Nod1 ligands actually needed to get access to the host cytosol to trigger cellular responses, or if trafficking and targeting to a specific subcellular compartment were the only requirement for Nod1-dependent activation. To answer this question, we pulsed HEK293T cells with iE-DAP, Tri-DAP or M-Tri-DAP for 10 min in the presence of digitonin, a membrane-permeabilizing toxin, then replaced back the cell culture medium to neutralize the action of digitonin, and lysed cells 6 h post-stimulation. Interestingly, in this experimental setting, all Nod1 ligands potently stimulated cells (Fig. 1E), therefore showing that presentation to the host cytosol is the limiting factor for their activating capacities. It must be noted, however, that in this context, iE-DAP remained a weaker agonist than Tri-DAP or M-Tri-DAP (Figure 4.1E), which correlates well with our previous observations using a different experimental setting (Girardin et al., 2003c), and may reflect a difference in the intrinsic capacity of this dipeptide to trigger Nod1, as compared with the naturally occurring tripeptide forms. Together, these results show that epithelial cells have the capacity to internalize Nod1 ligands, that these molecules display different trafficking abilities, and demonstrate that these agonists must reach the host cytosol to trigger Nod1-dependent responses.
A

\[
\begin{align*}
\text{D-Glu} & \quad \text{mesoDAP} \\
\text{L-Ala} & \quad \text{mesoDAP} \\
\text{Maramyl} & \quad \text{mesoDAP} \\
\text{iE-DAP} & \quad \text{Tri-DAP} \\
\text{M-Tri-DAP} & \quad \text{C12-iE-DAP} \\
\text{MDP} &
\end{align*}
\]

B

\[
\begin{align*}
\text{NF-κB (10 RLU)} & \\
\text{NS} & \quad 0.1 & \quad 1 & \quad 10 \\
\text{iE-DAP} & \quad 0.1 & \quad 1 & \quad 10 \\
\text{Tri-DAP} & \quad 0.1 & \quad 1 & \quad 10 \\
\text{M-Tri-DAP} & \quad 0.1 & \quad 1 & \quad 10 \\
\end{align*}
\]

C

\[
\begin{align*}
\text{NF-κB (10 RLU)} & \\
\text{NS} & \quad 2 & \quad 3 & \quad 4 & \quad 6 \\
\text{Tri-DAP} & \quad 2 & \quad 3 & \quad 4 & \quad 6 \\
\text{M-Tri-DAP} & \quad 2 & \quad 3 & \quad 4 & \quad 6 \\
\end{align*}
\]

D

\[
\begin{align*}
\text{NF-κB (10 RLU)} & \\
\text{NS} & \quad 0.1 & \quad 1 & \quad 10 \\
\text{iE-DAP} & \quad 0.1 & \quad 1 & \quad 10 \\
\text{C12-iE-DAP} & \quad 0.1 & \quad 1 & \quad 10 \\
\end{align*}
\]

E

\[
\begin{align*}
\text{NF-κB (10 RLU)} & \\
\text{NS} & \quad \text{DB} & \quad \text{DB} \\
\text{iE-DAP} & \quad \text{DB} & \quad \text{DB} \\
\text{Tri-DAP} & \quad 1 & \quad 5 \\
\text{M-Tri-DAP} & \quad 1 & \quad 5 \\
\end{align*}
\]

+ Digitonin (10%)
**Figure 4.1. Differential entry of Nod1 ligands into HEK293T cells.**

A, Nod1- and Nod2-activating molecules used in this study. The four Nod1-activating molecules contain the minimal motif D-Glu-mesoDAP (iE-DAP) that triggers Nod1. C12-iE-DAP contains a fatty acid moiety (lauroyl group) linked to the D-Glu. MDP stimulates Nod2 activity. All these molecules are synthetic and therefore do not contain any microbial contaminant. B, HEK293T cells were transfected overnight with the NF-κB reporter plasmid Igκ-luci. The following day, increasing concentrations of Nod1 ligands iE-DAP, Tri-DAP or M-Tri-DAP were added to the cell culture medium for 6h before cell lysis. C, HEK293T cells were transfected with Igκ-luci overnight and Tri-DAP or M-Tri-DAP were added to the cell culture medium for 1–6h on the following day, as indicated. D, same as B, but comparing the responses to increasing concentrations of iE-DAP and C12-iE-DAP. E, HEK293T cells were transfected overnight with Igκ-luci in DMEM cell culture medium. The following day, cell culture medium was replaced to an isotonic digitonin buffer (DB) and iE-DAP, Tri-DAP or M-Tri-DAP (all at either 1 μg/ml or 5 μg/ml) were added to the cells in the presence of digitonin for 10 min. Then, cell culture medium was replaced to DMEM for 6h, before cell lysis. NS, non stimulated.
We next investigated the cellular pathways responsible for the internalization of Nod1 ligands into HEK293T cells. Nocodazole and cytochalasin D, two drugs that block macropinocytosis by disrupting microtubules and actin cytoskeleton, respectively, did not affect the capacity of Tri-DAP or M-Tri-DAP to stimulate cells (Figure 4.2A). In contrast, Dynasore, a highly specific inhibitor of dynamin (Macia et al., 2006), potently blocked Tri-DAP- and M-Tri-DAP-dependent activation of NF-κB (Figure 4.2A). We next took advantage of the fact that Dynosore blocks dynamin in seconds or minutes (Macia et al., 2006), to study further the kinetics of dynamin-dependent entry of Nod1 ligands into cells, by adding the drug at various times before or after the addition of Nod1 ligand. Using this technique, we observed that the endocytosis of Nod1 ligands is a slow and continuous process that occurs in a linear fashion over time (Figure 4.2B). Because dynamin is known to be crucial for both clathrin-coated and caveolae vesicular endocytic pathways (Drubin et al., 2005; Kaksonen et al., 2006), we aimed to distinguish between these two potential entry mechanisms. Intracellular potassium depletion specifically blocks clathrin-dependent endocytosis (Drubin et al., 2005; Kaksonen et al., 2006). Therefore, we dissipated K⁺ gradients by treating cells with valinomycin, a K⁺ ionophore, and observed that this drug potently blunted the cellular response to Nod1 ligands (Figure 4.2C). Moreover, we used methyl-β-cyclodextrin (MβCD), a molecule that alters the fluidity of the plasma membrane by depleting cholesterol, and known to perturb caveolae vesicular endocytic pathways (Kaksonen et al., 2006). Strikingly, we observed that MβCD actually potentiated the cellular response to Nod1 ligands (Figure 4.2D), thus excluding a role for caveolae in mediating the entry of Nod1 ligands into HEK293T cells. Together, these results identify clathrin-dependent endocytosis as the mode of entry of Nod1 ligands into HEK293T epithelial cells.
Figure 4.2 Nod1 ligands enter HEK293T cells by endocytosis.

In all experiments below, HEK293T cells were first transfected overnight with Igκ-luci. A, cells were stimulated for 6h with 5 μg/ml Tri-DAP or M-Tri-DAP in the absence or presence of the cellular trafficking inhibitors nocodazole (Noc; 1μg/ml), cytochalasin D (CyD; 1 μg/ml) and Dynasore (Dyn; 80 μM). B, cells were stimulated for 6h with 5 μg/ml Tri-DAP, in the presence or absence of dynasore (80 μM) added either prior to the Nod1 ligand (-30'), or at various times after the addition of Tri-DAP, as indicated. C, cells were stimulated for 6h with 5 μg/ml Tri-DAP in the absence or presence of valinomycin (Valino; 1 μM or 10 μM). D, cells were stimulated for 6h with 1 μg/ml Tri-DAP in the absence or presence of methyl-β-cyclodextrin (MβCD; 2.5 mM or 10 mM). NS, non stimulated.
Endosomal maturation results in progressive acidification of the vesicular lumen, through a series of events, involving first the action of proteins such as the Na\(^+\)/H\(^+\) exchangers, followed by further acidification mediated by the V-ATPase complex in late endosomes and lysosomes (Steinberg et al., 2007). We aimed to identify if endosomal acidification played a role in the ability of Nod1 ligands to get exported to the cytosol. Cells treated with carbonyl cyanide m-chlorophenylhydrazone, a H\(^+\) ionophore, displayed blunted responses to Nod1 ligands (Figure 4.3A), thus suggesting that cellular proton gradients are critical for the trafficking and/or internalization of Nod1 ligands into host cytosol. Using bafilomycin A1, a specific inhibitor of V-ATPases, we observed that the acidification induced by this pump was not required for optimal trafficking of Nod1 ligands (Figure 4.3B), therefore showing that these agonists likely exit the endocytic pathway in early/sorting endosomes. Of note, we noticed that bafilomycin A1 actually increased cellular responses to Nod1 ligands, which might be explained by the fact that early endosomes, stalled at a mildly acidified stage, are blocked at a pH close to the one optimal for transport of Nod1 ligands out of the endocytic machinery.
Figure 4.3. The internalization of Nod1 ligands is dependent on endosomal pH.

In the experiments below, HEK293T cells were first transfected overnight with Igκ-luci. A, cells were stimulated for 6h with 5 μg/ml Tri-DAP in the absence or presence of carbonyl cyanide m-chloro phenylhydrazone (CCCP; 1–10 μM). B, cells were stimulated for 6h with 5 μg/ml Tri-DAP or M-Tri-DAP in the absence or presence of bafilomycin A1 (Baf; 10–50 nM). NS, non-stimulated.
Our last results suggested that an optimal luminal pH might exist for the efficient transport of Nod1 ligands to the cytosol. In order to study this question further, we developed a procedure, relying on the transient (60 min) acidification of the extracellular milieu in an isotonic buffer in the presence of Nod1 ligands (Figure 4.4A). Strikingly, we observed that, although this procedure did not affect clathrin-mediated endocytosis in general (Figure 4.4B), it altered greatly the cellular response to the Nod1 ligand iE-DAP. In particular, we identified that an optimal pH for triggering Nod1-dependent responses existed, and was around pH 5.5–6 (Figure 4.4C), which is a level of acidification commonly observed in early endosomes (Steinberg et al., 2007). The fact that, through this experimental procedure, we could observe considerable cellular activation by iE-DAP reinforces the idea that this ligand is not intrinsically a poor Nod1 ligand, but rather is impaired in its capacity to reach endosomes acidified to 5.5–6. Through titration (Figure 4.4D) and kinetics (Figure 4.4E) experiments, we refined our observations and noticed that as little as 1 μg/ml of iE-DAP, and a transient extracellular acidosis for 20 min or more, induced measurable iE-DAP-dependent cellular responses. More importantly, we demonstrated that the procedure of transient extracellular acidosis that we developed likely bypassed normal endosomal maturation, but still relied on dynamin-dependent pinching off of endosomes from the plasma membrane, as it was fully blocked by Dynasore (Figure 4.4F). Next, we used this procedure of transient extracellular acidosis to compare the relative capacities of distinct Nod1 ligands to trigger cellular NF-κB responses. We first noticed that the tripeptide ligand Tri-DAP displayed more potent activating capacities than iE-DAP (Figure 4.4G), in agreement with our previous experiments using different experimental systems (see above). Surprisingly, we observed in this experimental setting that M-Tri-DAP was almost completely unable to trigger cellular responses (Figure 4.4G), while M-Tri-DAP and Tri-DAP displayed similar activating capacities in normal stimulating conditions (see Figure 4.1B). These results suggest that a processing of M-Tri-DAP into Tri-DAP likely occurs in normally maturing endosomes, which is bypassed in our experimental system where endosomal acidification is artificially provoked by transient acidosis (see also below Figure 4.9). These observations also provide evidence that the exit of Nod1 ligands from early endosomes is a process that is sterically gated, thus indirectly suggesting the existence of a specific transport system for Nod1 ligands within the early endosome.
Figure 4.4. Extracellular acidification strongly potentiates endocytosis-mediated entry of iE-DAP.

A. schematic representation of the experimental procedure followed to study the influence of extracellular pH on NF-κB activation by Nod1 ligand iE-DAP. The acidification was maintained for only 1h, in a specific isotonic buffer (IB), a period when the ligand was added. Cell medium was replaced to DMEM, pH 7, for the last 5h, before cell lysis. B, uptake of transferrin-Alexa Fluor 568 (10 μg/ml) by HEK293T cells for 1h in IB buffer pH 7 (left) or pH 5.5 (right) was followed by fluorescence microscopy. Overlay with differential interference contrast is also shown. For the experiments below (C–G), HEK293T cells were first transfected overnight with Igκ-luci, and transient acidosis in IB was performed, as described in A. C, cells were stimulated for 1h with iE-DAP (10 μg/ml) in IB buffer at different pH, as indicated. D, Cells were stimulated for 1h with various concentrations of iE-DAP (0.1 to 10 μg/ml) in IB buffer pH 5.5. E, cells were stimulated for various times (0–60 minutes) with iE-DAP (10 μg/ml) in IB buffer, pH 5.5. F, cells were stimulated for 1h with iE-DAP (5 μg/ml) in IB buffer, pH 5.5, in the presence or absence of Dynasore (Dyn; 80 μM). G, cells were stimulated for 1h with iE-DAP (5 μg/ml), Tri-DAP (1 μg/ml) or M-Tri-DAP (1 μg/ml) in IB buffer, pH 5.5. NS, non-stimulated.
We then aimed to identify if the internalization of the Nod2 ligand, MDP, displayed similar characteristics than that of Nod1 ligands in HEK293T cells. When added extracellularly for 2–6 h, we observed that MDP triggered only minimal NF-κB-dependent cellular responses (Figure 4.5A), which is in agreement with previous reports on Nod2-dependent responses in epithelial cells (Girardin et al., 2003a). Indeed, it has been reported by several groups that the levels of Nod2 expression in most epithelial cells, including HEK293T, is minimal or absent in normal conditions (Gutierrez et al., 2002; Rosenstiel et al., 2003). Therefore, we co-transfected overnight minimal amounts of a Nod2 expressing vector (0.2 ng) together with our Igκ-luci reporter construct, and then stimulated cells with MDP for increasing periods the following day. Using this procedure, we noticed an increase in the overall sensitivity of HEK293T cells to MDP (Figure 4.5B), which prompted to select this experimental setting for further studies. Next, we observed that MDP, like Nod1 ligands, entered cells through dynamin-dependent and caveosome-independent endocytosis (Figure 4.5, C and D), and that the export out of the endocytic machinery occurred prior to acidification dependent on the action of the V-ATPase, as it was not blocked by bafilomycin A1 (Figure 4.5C). Finally, using the transient extracellular acidosis procedure described above, we observed that MDP internalization also required an optimal pH in HEK293T cells, but that it exhibited a broader range of pH 5.5 to 6.5 (Figure 4.5E) than that of Nod1 ligands.
Figure 4.5. The Nod2 ligand MDP enters HEK293T cells by endocytosis.

In all experiments below, HEK293T cells were first transfected overnight with Igκ-luci. A, cells were stimulated for various times (2–6 h) with muramyl dipeptide (MDP; 10 μg/ml). For the next experiments (B–E), cells were co-transfected with an expression vector encoding for hNod2 (0.2 ng/well). B, cells were stimulated for various times (2–6 h) with MDP (10 μg/ml). C and D, cells were stimulated for 6 h with MDP (10 μg/ml) in the presence or absence of bafilomycin A1 (Baf; 50 nM), Dynasore (Dyn; 80 μM) (C) or (D) MβCD (2.5 mM or 10 mM) (D). E, cells were stimulated for 1h with MDP (10 μg/ml) in IB buffer at different pH, similarly to Figure 4.4C. NS1, non-stimulated; NS2, non-stimulated but overexpressing hNod2 (0.2 ng/well).
Next, we aimed to identify the nature of the putative transporter, expressed in early endosomes, which was responsible for the sterically gated internalization of Nod1 ligands to the cytosol. Out of the 46 families of solute carrier (SLC) proteins, only SLC15 proteins are known to transport di- or tri-peptides. Moreover, SLC15 proteins co-transport oligopeptides together with H\(^+\) and have an optimal pH for transport around pH 5.5–6 (Daniel and Kottra, 2004). Therefore, we reasoned that the putative transporter for Nod1 ligands in HEK293T cells might be a member of the SLC15 family. We first analyzed the expression of SLC15 proteins in HeLa, HEK293T and MCF-7 human epithelial cell lines by semi-quantitative PCR. The SLC15 family is composed of four members SLC15A1 to SLC15A4 in mammals. SLC15A3 was not detected in these samples and SLC15A1 was expressed at low levels in HEK293T cells but not HeLa or MCF-7 (Figure 4.6A). In contrast, transcripts for SLC15A2 and SLC15A4 were strongly expressed in the three cell lines tested (Figure 4.6A). We first investigated the potential role of SLC15A2 in mediating the transport of Nod1 ligands by using Lys[Z(NO\(_2\)]-Val, a highly specific inhibitor of this transporter (Ki=0.097 \(\mu\text{M}\)) (Biegel et al., 2006). Even at the highest non-cytotoxic dose tested (50 \(\mu\text{M}\)), the inhibitor failed to inhibit Nod1 ligand-mediated NF-\(\kappa\)B activation (Figure 4.6B), therefore suggesting that SLC15A2 was not required in our experimental system. Similarly, cefadroxil, an antibiotic that inhibits both SLC15A1 and SLC15A2 (Biegel et al., 2006), had no effect on Nod1 ligand-mediated NF-\(\kappa\)B activation (data not shown). We then hypothesized that SLC15A4 could be implicated in the transport of Nod1 ligands. A polyclonal antibody raised against human SLC15A4 revealed multiple forms both at the endogenous level and in SLC15A4-V5 overexpressed cells, migrating at \(~60\) kDa and higher (Figure 4.6C), suggesting the existence of extensive post-transcriptional modifications of SLC15A4 in unstimulated conditions. Similar results were obtained using another anti-SLC15A4 polyclonal antibody (data not shown). Next, we tested a number of siRNA constructs designed to knock down endogenous SLC15A4 expression by transient transfection, and identified duplex B as the most effective one (Figure 4.6D), which was used for functional studies. Interestingly, lentiviral-mediated knockdown of SLC15A4 in HEK293T cells resulted in significant decrease of NF-\(\kappa\)B activation by the Nod1 ligands Tri-DAP and C12-iE-DAP in luciferase assay (Figure 4.6E), thus demonstrating that SLC15A4 was critical for Nod1 ligand-mediated signaling in HEK293T cells. In the case of MDP, the results were inconclusive because of the low level of NF-\(\kappa\)B activation triggered by this muramyl peptide in our experimental system.
Figure 4.6. SLC15A4 is involved in Nod1 ligand-mediated NF-κB activation in HEK293T cells.

A, the expression of SLC15 was determined by RT-PCR in HeLa, HEK293T and MCF-7 human epithelial cell lines. B, HEK293T cells were transfected overnight with Igκ-luci, and transient acidosis in IB was performed. Cells were stimulated for 1 h with M-Tri-DAP (1 μg/ml) in IB buffer pH 5.5 in the presence or absence of increasing concentration of the SLC15A2 inhibitor Lys[Z(NO$_2$)]-Val. C, HEK293T cells were transfected with increasing concentration of SLC15A4-V5 expression vector, and cell lysates analyzed by western blotting using anti-SLC15A4 antibody. * indicates a non-specific band. D, HEK293T cells were transfected for 72 h with various siRNA duplexes (constructs A–F) against human SLC15A4 or a control non-targeting siRNA duplex (Ctr siRNA) and cell lysates analyzed by western blotting using anti-SLC15A4 antibody. * indicates a nonspecific band. The membrane was stripped and blotted against tubulin for loading control. E, HEK293T cells transduced for 72 h with lentiviral particles expressing shRNAs against SLC15A4 or a non-targeting sequence were transfected overnight with Igκ-luci and stimulated for 1 h with Nod1 ligands (iE-DAP, Tri-DAP, M-Tri-DAP) or Nod2 ligand MDP in acidosis conditions (IB pH 5.5), or with C12-iE-DAP normal (non-acidified) conditions, and luciferase activity was measured 6 h post-stimulation in cell lysates. Statistical analysis was performed using a two-tailed unpaired t test, and conditions for which p<0.05 were IB, pH 5.5/Tri-DAP (p=0.002) and C12-iE-DAP (p=0.035). NS1, non-stimulated; NS2, non-stimulated in IB, pH 5.5.
We next investigated the subcellular localization of SLC15A4 by immunofluorescence in HEK293T cells. First, we observed that over-expressed SLC15A4-V5 colocalized with Rab5-RFP, thus showing that this transporter was expressed in early endosomes (Figure 4.7A). Interestingly, transient extracellular acidosis (pH 5.5), similar to the procedure performed above in luciferase assays, resulted in elongation of the cells and relocalization of SLC15A4 to the cellular poles (Figure 4.7B), where it partially colocalized with Rab5 (Figure 4.7C). It is possible that the cellular redistribution of SLC15A4 in early endosomes at mildly acidic pH may contribute to the enhancement of Nod1-dependent signaling that was observed in luciferase assays.

Recent studies have linked IBD to either polymorphisms in genes encoding SLC15 family members (Zucchelli et al., 2009) or changes in the intestinal expression of SLC15 proteins (Wojtal et al., 2009). Because of the implication of Nod1 and Nod2 in IBD, we aimed to identify if the mRNA expression levels of SLC15A4 was altered in the intestine of IBD patients. We analyzed by real-time PCR the expression of SLC15A4 mRNA in colonic biopsies from 53 ulcerative colitis (UC) and 49 Crohn’s disease (CD) patients, and inflamed versus non-inflamed sections were analyzed separately. For CD patients, inflamed versus non-inflamed sections of the terminal ileum were also analyzed. Interestingly, SLC15A4 expression was significantly upregulated in inflamed areas of the colon in CD ($p<0.05$) and UC ($p<0.001$) patients, but not in the terminal ileum of CD patients (Figure 4.8).
Figure 4.7. SLC15A4 is expressed in early endosomes.

A, HEK293T cells were transfected with SLC15A4-V5 and Rab5-RFP and visualized by immunofluorescence using an anti-V5 antibody. B, HEK293T cells were transfected with SLC15A4-V5, cultured for 1 h in IB pH 7 or pH 5.5, before fixation, and visualized by immunofluorescence using an anti-V5 antibody. C, HEK293T cells were transfected with SLC15A4-V5 and Rab5-RFP were cultured for 1 h in IB pH 5.5, before fixation, and visualized by immunofluorescence using an anti-V5 antibody. Arrows indicate localization of SLC15A4 and Rab5-RFP at cellular poles.
Figure 4.8. *SLC15A4* mRNA expression levels are increased in inflamed colon of IBD patients.

*SLC15A4* expression levels in biopsies from colon and terminal ileum of CD and UC patients were determined by absolute quantification of SLC15A4 copy numbers and normalized to mRNA copy numbers of villin. The bars represent average values of mRNA expression levels of subgroups of IBD patients. Error bars represent SD. *, $p<0.05$; ***, $p<0.0001$. 
4.5 Discussion

The results that we have presented here establish that, in epithelial cells, muramyl peptides get internalized and traffic via the endocytic machinery, and most likely rely solely on the clathrin-dependent coated pit pathway. This entry pathway is the one generally used for receptor-mediated endocytosis, and this raises the possibility that a cell surface receptor might be involved in the optimal uptake of either muramyl peptides or peptidoglycan-derived di- or tripeptides. In such a scenario, the interaction of these Nod agonists with the cell surface could be either achieved through specific recognition of a peptidic motif by a receptor, or occur nonspecifically via electrostatic interactions. It must be noted that our results demonstrated that iEDAP and Tri-DAP trigger cellular responses in a very different fashion, and that this likely results from an inability of iEDAP to travel down the endocytic pathway to mildly acidified endosomes. It is possible that these observations reflect a differential capacity of these peptides to form interactions with cell surface receptors/proteins, prior to the invagination of the endocytic cup.

Our results showing that Nod ligands are internalized into host cells via endocytosis are consistent with a number of indirect observations of the biological activity of these molecules. In particular, we and others have repeatedly shown that the efficiency of Nod ligands is greatly boosted by “co-transfecting” those ligands overnight together with plasmidic DNA into cationic liposomes (Girardin et al., 2003b; Inohara et al., 2003). Because liposome-DNA complexes are known to traffic mostly via receptor-mediated endocytosis, these initial observations suggested that: (i) the entry of Nod ligands into host cells can be potentiated if these molecules traffic through this pathway and (ii) endocytosis is likely the most critical limiting factor for the stimulating capacities of Nod-activating molecules. In agreement with this, early studies aiming at increasing the stimulating capacities of peptidoglycan-derived peptides or muramyl peptides demonstrated that the covalent addition of lipophilic groups to these structures could greatly enhance cellular responses. For instance, the tumoricidal activity of a lipophilic derivative of MDP on endothelial cells has been found to be enhanced 100- to 1000-fold as compared to MDP (Phillips et al., 1994). Moreover, studies with 6-O-acyl derivatives of MDP revealed that 6-O-(2-tetradecylhexadecanoyl)-MDP and 6-O-(3-hydroxy-2-tetradecyl-octadecanoyl)-MDP exhibited stronger macrophage-stimulating effects than MDP (Takada et al., 1979). Together, a number of convergent studies demonstrated that delivering muramyl peptides via liposomal or fatty acid-
containing formulations greatly improved the immunogenicity and adjuvanticity of these bacterial molecules (Fogler and Fidler, 1987; Mehta et al., 1982; Nayar et al., 1986; Schroit and Fidler, 1982). Therefore, understanding how Nod ligands enter into cells will help design novel rational strategies aiming at increasing their biological activity. Moreover, it must be noted that in natural conditions, muramyl peptides are rarely found free in the extracellular milieu, and are commonly associated with other bacterial cell wall components that carry fatty acid groups. These include lipoproteins, lipoteichoic acid, mycolic acids or LPS, and might play a crucial role in the potentiation of endocytosis-mediated entry of Nod ligands.

A recent report has demonstrated that MDP was internalized by macrophages through clathrin-dependent endocytosis (Marina-Garcia et al., 2009), which supports our findings in HEK293T cells. However, MDP entry in macrophages appeared to require V-ATPase-dependent endosomal acidification, contrasting with our observations in HEK293T cells. This might be due to differences in the endocytic trafficking machineries of phagocytic versus non-phagocytic cells. Alternatively, it is possible that macrophages and epithelial cells rely on different transporters for Nod ligands, which would be expressed in distinct sub-cellular compartments, such as early endosomes or lysosomes. Indeed, a number of muramyl peptide transporters could exist and be differentially expressed in specific cell types and/or sub-cellular compartments. This hypothesis is supported by the fact that SLC15A1 was identified as an MDP transporter in Caco-2/bbe epithelial cells (Vavricka et al., 2004), but found to be dispensable for mediating MDP entry in macrophages (Marina-Garcia et al., 2009). Similarly, SLC15A2 has been shown to trigger transport of the Nod1 ligand iE-DAP in human upper airway epithelial cells (Swaan et al., 2008), but was found to be dispensable in our assays. Finally, it must be noted that the knockdown of SLC15A4 expression in HEK293T cells did not result in a complete ablation of NF-κB activation induced by Nod1 ligands. This suggests that other transporters might contribute to the transport of these peptidoglycan-derived peptides in HEK293T cells and possibly other cell populations.

An interesting question that emerges from our studies is to determine if the limiting factor for Nod-dependent activation is solely the cytosolic presentation of its ligands, or if endocytosis in itself participates in driving NF-κB-dependent activation of Nod1 and Nod2. Indeed, an argument in favor of the latter hypothesis comes from the fact that membrane targeting of Nod2
is required for its ability to trigger NF-κB (Barnich et al., 2005). Moreover, studies on TLR4 argue that the subcellular localization of this protein to early endosomes is required to drive type I interferon responses, through the specific engagement of the adaptor protein TRAM (Kagan et al., 2008). However, our results demonstrate that the sole presentation of Nod ligands to the host cytosol (using the membrane permeabilizing toxin digitonin) is sufficient to recapitulate Nod-dependent activation of NF-κB. This strongly suggests that endocytosis of Nod ligands is the most efficient means for allowing access of these molecules to the cytosol, but appears dispensable, provided that these molecules could access the cytosol through other means. This observation is of importance since it implies that Nod proteins could detect free muramyl peptides generated by pathogenic bacteria that would have gained access to the host cytosol, such as *Shigella* or *Listeria*, long after these bacteria would have ruptured the phagosome.

Our results demonstrated that an optimal luminal pH was required for efficient delivery of muramyl peptides into the cytosol, and this correlates with the identification of SLC15A4 as a transporter for Nod1 ligands in early endosomes. Indeed, SLC15 proteins have been shown to display optimal transport properties at mildly acidic pH (ranging from 5.5 to 6) (Daniel and Kottra, 2004). We have also observed that the transport of Nod1 ligands from early endosomes was sterically gated, as iE-DAP and Tri-DAP, but not M-Tri-DAP, were efficiently internalized in our experimental setting of transient acidosis. Again, these results are in agreement with the current knowledge on the steric constraints for SLC15-mediated transport of oligopeptides; indeed, studies on SLC15A1 (Fei et al., 1994) and SLC15A2 (Boll et al., 1996) have demonstrated that these transporters have a much greater affinity for di- or tripeptides than other oligopeptides. In this regard, cleavage of the muramyl group by endosomal hydrolytic enzymes such as PGRP-L/PGLYRP2 might represent a prerequisite to SLC15A4-mediated transport of Nod1 ligands to the cytosol. Consequently, we hypothesize that naturally-occurring muramyl peptides (such as M-Tri-DAP) are likely processed by endosomal hydrolytic enzymes, which are progressively acquired during normal endosomal maturation (Figure 4.9). Therefore, the lack of some critical enzymes from our artificially acidified endosomes likely accounts for the incapacity to process M-Tri-DAP adequately.

Together, our observations characterized the mechanism by which Nod ligands access the host cytosol in human epithelial cells, and demonstrated a crucial role for endosomal uptake and pH-dependent transport of these molecules to the cytosol by SLC15A4. Our results provide new
avenues for the development of therapeutic strategies aiming at either improving the uptake of muramyl peptides, or targeting the pathway responsible for the cytosolic transport of these bacterial molecules. A better understanding of how Nod proteins detect intracellular bacterial peptidoglycan will provide insights into the etiology of inflammatory diseases such as asthma and Crohn’s disease, which are associated with mutations in *Nod1* and *Nod2*. 
Figure 4.9. Schematic representation of the model for the entry of Nod1 ligands into epithelial cells.

The normal entry pathway for Nod1 ligands is presented on the left, while extracellular acidosis-induced entry is displayed on the right. We speculate that acidosis-mediated entry allows bypassing the progressive acidification normally observed in maturing endosomes, resulting in a different vesicular content. In particular, our results suggest that the putative M-Tri-DAP hydrolase, which is normally present in early endosomes, would be absent from experimentally acidified endosomes.
Chapter 5
Synthesis and Biological Evaluation of Biotinyl Hydrazone Derivatives of Muramyl Peptides

This chapter was published as


*These authors contributed equally to this work.

Contribution of Data

All experiments/analyses were performed by Jooeun Lee, unless noted otherwise;

Figure 1 & Table 1: all of the preparation and designing hydrazone derivatives were performed by Didier Blanot.
5

5.1 Abstract

Muramyl peptides derived from bacterial peptidoglycan have long been known for their ability to trigger host innate immune responses, including inflammation and antimicrobial defense. Muramyl peptides have also been widely studied for their role as immune adjuvants. In mammals, the nucleotide-binding oligomerization domain (Nod) proteins Nod1 and Nod2 detect distinct muramyl peptide structures and mediate their biological activity. Because of the poor immunogenicity of these small peptidoglycan derivatives, research in this field is currently limited by the lack of reagents to track or immobilize specific muramyl peptides. We present here the generation and initial biological characterization of synthetic muramyl peptides covalently coupled to dansyl or biotinyl derivatives, and demonstrate that biotinyl coupling on the muramyl moiety results in derivatives that can be tracked by immunofluorescence and maintain full biological activity, as observed by their capacity to trigger Nod signaling. Moreover, using digitonin-mediated permeabilization techniques on live cells, we also demonstrate that biotinylated muramyl peptides efficiently reach the host cytosol, where they activate Nod signaling. Therefore, these derivatives represent useful probes to study the cell biology and the biochemistry of host responses to muramyl peptides.

5.2 Introduction

It is now well established that the innate immunity system acts through detection of microbial motifs known as PAMPs (pathogen-associated molecular patterns) (Philpott and Girardin, 2004). Among these, peptidoglycan and its hydrolysis products, muramyl peptides, are sensed by the intracellular Nod proteins Nod1 and Nod2. While Nod2 is a general sensor for all peptidoglycans since it recognizes the common muramyl dipeptide (MurNAc-L-Ala-D-Glu) motif (Girardin et al., 2003b; Girardin et al., 2003c; Inohara et al., 2003), Nod1 senses the γ-D-Glu-meso-A₂pm dipeptide motif found in the peptidoglycan of most Gram-negative bacteria, and the meso-A₂pm amino acid must be in terminal position for Nod1-mediated detection (Chamaillard et al., 2003; Girardin et al., 2003a; Girardin et al., 2003c). Following detection of specific muramyl peptides, Nod proteins trigger a wide array of inflammatory, antimicrobial and immune adjuvant responses (Geddes et al., 2009). Using indirect approaches, we and others recently demonstrated that muramyl peptides are efficiently internalized by mammalian cells through clathrin-mediated
endocytosis (Lee et al., 2009; Marina-Garcia et al., 2009). However, the lack of suitable labeled muramyl peptides prevents direct analysis of their trafficking by immunofluorescence. In the present work, we have synthesized and used derivatives consisting of these compounds linked with a dansyl (Dns) or a biotinyl-6-aminohexanoyl (Bio-Ahx) probe through a hydrazone functionality. Dansylated muramyl peptides did not display strong enough emission capacity for the use in immunofluorescence on live cells. By contrast, biotinylated muramyl peptides with the biotinyl group covalently linked to the muramyl group were endowed with interesting properties since i) they demonstrated good biological activity for both Nod1 and Nod2 agonists in luciferase assays; ii) in association with streptavidin-coupled fluorochromes, they proved to display sensitivity compatible with their use in immunofluorescence. Using digitonin-mediated permeabilization techniques on live cells, we also demonstrated that biotinylated muramyl peptides efficiently reach the host cytosol, where they activate Nod signaling. Together, biotinylated muramyl peptides appear to display an interesting versatility for their use in different cellular assays. These molecules will therefore prove to be useful for studying the cell biology and biochemistry of Nod signaling.

5.3 Materials & Methods

Compounds. Dansyl hydrazine (Dns-NHNH$_2$), N-biotinyl-6-aminohexanoyl hydrazine (Bio-Ahx-NHNH$_2$), and L-Ala-$\gamma$-D-Glu-meso-A$_2$pm (Tri-A$_2$pm) were purchased from Fluka, Pierce, and InvivoGen, respectively.

Muramyl peptides. MurNAc and MDP were purchased from Sigma and InvivoGen, respectively. MurNAc-L-Ala and MurNAc-L-Ala-D-Glu were synthesized according to the published procedures (Kusumoto et al., 1976; Lefrancier et al., 1977). The other muramyl peptides were obtained from the corresponding UDP-MurNAc-peptides (Babic et al., 2007) by mild acid hydrolysis (0.1 M HCl, 100°C, 10 min) (Girardin et al., 2003c). The residue resulting from the evaporation of the reaction mixture was used without purification for hydrazone formation.

Synthesis of dansyl hydrazones. To muramyl peptide (100 nmol) dissolved in water (10 µl), 3% (w/v) trichloracetic acid (10 µl) and 1% (w/v) Dns-NHNH$_2$ in acetonitrile (20 µl) were added. The mixture was stirred for 20 min at 65°C and evaporated in vacuo. The product was purified by RP-HPLC.
**Synthesis of biotinylated hydrazones.** To dry muramyl peptide (150 nmol), 7.5 mM Bio-Ahx-NHNH₂ in 30% acetonitrile (100 µl) were added. The mixture was evaporated to dryness, then dissolved in methanol/water/acetic acid 95:4:1 (50 µl; v/v). The mixture was stirred overnight at 60°C and evaporated in vacuo. The product was purified by RP-HPLC.

**Purification of hydrazone derivatives.** The reaction mixture was taken up in 20% (v/v) methanol and injected onto a column (250 × 4.6 mm) of Econosphere C₁₈ (5 µm; Alltech France) equipped with a guard column of ODS-Hypersil (Thermo-Fisher Scientific). Elution was performed at a flow rate of 0.6 ml.min⁻¹ with a gradient of methanol (elucent A: 20 mM ammonium acetate in water/methanol 4:1; eluent B, 20 mM ammonium acetate in water/methanol 1:4; gradient: 0% B from 0 to 10 min, 0 to 60% B from 10 to 50 min, 60 to 100% B from 50 to 55 min, 100% B from 55 to 60 min). The compounds were detected at either 247 nm (Dns derivatives) or 220 nm (Bio-Ahx derivatives). Peaks were collected manually, evaporated, taken up in water and lyophilized. The compounds were finally dissolved in water/methanol 2:1 (225 µl; v/v) and stored at -20°C.

**Amino acid analysis.** Samples were hydrolyzed in 6 M HCl at 95°C for 16 h. After evaporation of the acid, the hydrolyzates were dissolved in 67 mM trisodium citrate-HCl (pH 2.2) and injected into a Hitachi L8800 analyzer equipped with a 2620MSC-PS column (ScienceTec).

**MALDI-TOF mass spectrometry.** Positive spectra were recorded in the reflectron mode with delayed extraction on a Perseptive Voyager-DE STR instrument (Applied Biosystems) equipped with a 337-nm laser. The compound (0.5 or 1 µl) was deposited on the plate, followed by 2,5-dihydroxybenzoic acid (1 µl at 10 mg.ml⁻¹ in 20 mM diammonium citrate). After evaporation of the solvents, spectra were recorded at an acceleration voltage of +20 kV and an extraction delay time of 200 ns. External calibration was performed using the calibration mixture of the Sequazime™ peptide mass standard kit (Applied Biosystems).

**Cell culture and reagents.** Human HEK293T and HeLa epithelial cell lines (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 5% fetal calf serum (FCS) and 1% penicillin/streptomycin. Cells were maintained in 95% air, 5% CO₂ at 37°C. Endotoxin-free FCS and phosphate buffer saline were from Wisent (St-Bruno, Quebec). FCS was used after heat inactivation at 56°C for 30 min. All
cell culture reagents and antibiotics were also from Wisent. Dynasore (3-hydroxy-naphthalene-2-carboxylic acid (3,4-dihydroxy-benzylidene)-hydrazide monohydrate) and digitonin were from Sigma.

**NF-κB activation assays.** Transfections were carried out using polyethylenimine (PEI, Polysciences Inc., Warrington, PA) in HEK293T according to the manufacturer’s instructions. Briefly, cells were transfected overnight with 75 ng of NF-κB luciferase reporter plasmid (Igk-luc, Invitrogen). The empty vector (pcDNA3, Invitrogen) was used to balance the transfected DNA concentration. The expression vector for human Nod2 (0.2 ng/well) was a kind gift from Dr. Núñez (University of Michigan, Ann Arbor, MI). Following transfection, biotinylated ligands were added the next day for 6 h (unless specified) before performing luciferase measurements. For NF-κB activation assays in digitonin-permeabilized cells, HEK293T were incubated for 10 min at 37°C with Nod1 ligands in isotonic digitonin buffer (Girardin et al., 2003a) (500 µl) with or without 10 µg/ml digitonin, and then placed in DMEM for 6 h. The dose of 50 nM was used for modified muramyl peptides because our previous results (Magalhaes et al., 2005) have demonstrated that this dose typically gives a non-saturated and close to maximal (usually from 50% to 100%) activation of Nod1 or Nod2. For positive controls (MDP and Tri-A₂pm), the dose of 10 µg/ml (ca. 20 µM) was used, as it typically provides maximal response in our assays.

**Immunofluorescence.** HeLa cells grown on glass coverslips were stimulated for 30 min with biotinylated muramyl peptide or biotinylated transferrin, as indicated. Next, 2 µg/ml Streptavidin-Alexa488 (Invitrogen) was added for 30 min. In some conditions, 1 µg/ml of directly coupled Transferrin-Alexa568 (Invitrogen) was added as a positive control, as indicated. Finally, cells were washed, fixed (4% paraformaldehyde, 15 min) and stained with DAPI to visualize nuclei. Images were taken using Zeiss Z-1 epifluorescence microscope with a 63× oil fluorescence objective and deconvolved using Volocity software (Quorum Technologies).
5.4 Results & Discussion

Muramyl peptides, obtained either by chemical synthesis or by mild acid hydrolysis of the corresponding UDP-MurNAc-peptides, were reacted either with Dns-NHNH₂ or Bio-Ahx-NHNH₂ (Fig. 1). For Dns hydrazones 1, 3 and 5, a procedure adapted from those of Mopper and Johnson (Mopper and Johnson, 1983) and Hull and Turco (Hull and Turco, 1985) was used. For Bio-Ahx hydrazones 2, 4 and 6-10, the method of Leteux et al. (Leteux et al., 1998) was followed. The compounds were purified by RP-HPLC. Theoretically, such hydrazones are mixtures of 4 isomers, two syn/anti isomers of the acyclic form and two α/β anomers of the cyclic form (Shinohara et al., 1995) (Fig. 1). As a matter of fact, the desired compound appeared as a dissymmetrical peak, or even as 2-3 overlapping peaks. The identity of the hydrazones was established by MALDI-TOF mass spectrometry: the positive-ion spectra displayed protonated or/sodiated molecular ions (M+H)+ and (M+Na)+ consistent with the calculated molecular masses (Table 1). Yields, determined by quantitative amino acid analysis, were in general moderate; they were higher for the biotinyl derivatives (21-95%) than for the dansyl ones (10-21%) (Table 1).

We next tested the biological activity of these muramyl peptides and, to do so, first performed luciferase assays in HEK293T cells. In this cellular system, HEK293T cells were first transfected overnight with the Igκ-luci reporter plasmid, which encodes for the luciferase gene whose expression is driven by NF-κB elements on its promoter. Along with the Igκ-luci plasmid, cells were transfected with expression vectors encoding either Nod1 or Nod2, in order to potentiate the cellular responses through these Nod-like receptor proteins. Next, cells were stimulated for 6 hours with 50 nM biotinylated muramyl peptides and lysed for luciferase analysis. We observed that only compound 7 (R¹= L-Ala-γ-D-Glu-meso-A₂pm) was able to stimulate Nod1-dependent responses (Fig. 2A), in agreement with the previously reported requirement of Nod1-activating muramyl peptides to contain a terminal meso-A₂pm (Chamaillard et al., 2003; Girardin et al., 2003a; Girardin et al., 2003c). Indeed, compound 9 (R¹= L-Ala-γ-D-Glu-meso-A₂pm-D-Ala-D-Ala), which also has a meso-A₂pm, but not in terminal position, was unable to trigger Nod1, as we previously observed (Girardin et al., 2003c). Compound 6 (R¹= L-Ala-D-Glu) was able to trigger Nod2-dependent responses (Fig. 2B), which is in agreement with the known capacity of MurNAc-L-Ala-D-Glu (Girardin et al., 2003c) or MurNAc-L-Ala-D-Glu-NH₂ (MDP)
Figure 5.1 Preparation of hydrazone derivatives 1-10. See Table 1 for the nature of $R^1$.
Table 5.1 Yields and mass spectrometry analysis of hydrazone derivatives

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<tr>
<th>Compound</th>
<th>R¹</th>
<th>R²</th>
<th>Yield (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Calculated&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Found&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
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<td>Bio-Alx</td>
<td>21</td>
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<td>1117.84</td>
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</table>

<sup>a</sup>Calculated from the amount of starting material (isolated muramyl derivative for compounds 1-6, or UDP-MurNAc-peptide for hydrazones 7-10) by quantitative amino acid analysis.

<sup>b</sup>Monoisotopic molecular mass.

<sup>c</sup>Main Peak.
Figure 5.2. Biotinylated and dansylated muramyl peptides are biologically active.

(A-B) HEK293T cells were transfected overnight with the reporter gene Igκ-luci to monitor NF-κB activation, together with either of the peptidoglycan sensors Nod1 (A) or Nod2 (B). The following day, cells were either left unstimulated or stimulated for 6 h with biotinylated muramyl peptides (compounds are the following: 2, Bio-Ahx-MurNAc; 9, Bio-Ahx-MurNAc-L-Ala-γ-D-Glu-meso-A2pm-D-Ala-D-Ala; 7, Bio-Ahx-MurNAc-L-Ala-γ-D-Glu-meso-A2pm; 6, Bio-Ahx-MurNAc-L-Ala-D-Glu), or with the positive controls Tri-A2pm (for Nod1, in (A)) or MDP (for Nod2, in (B)). (C) HEK293T cells were transfected overnight with Igκ-luci, together with Nod2. The following day, cells were either left unstimulated or stimulated for 6 h with dansylated muramyl peptides (compounds are the following: 1, Dns-MurNAc; 3, Dns-MurNAc-L-Ala; 5, Dns-MurNAc-L-Ala-D-Glu). All muramyl peptides used (biotinylated or dansylated) were added at the final concentration of 50 nM, and positive controls were at 10 μg/ml. NS, non stimulated.
(Girardin et al., 2003b; Inohara et al., 2003) to activate Nod2. Biotinylated compounds \(4 (R^1 = \text{L-Ala}), 8 (R^1 = \text{L-Ala-\(\gamma\)-D-Glu-L-Lys})\) and \(10 (R^1 = \text{L-Ala-\(\gamma\)-D-Glu-L-Lys-D-Ala-D-Ala})\) were unable to stimulate either Nod1 or Nod2 (data not shown), in keeping with our previous results on unmodified muramyl peptides (Girardin et al., 2003c). Moreover, in dose-response experiments, we noted that compounds \(7\) and \(6\) were able to activate Nod1 and Nod2, respectively, with efficiencies that were similar to the ones of the non modified muramyl peptides, thus showing that the addition of the biotinyl group to muramyl peptides does not significantly affect the stimulatory activity of these molecules (data not shown). Finally, we also noted that the dansylated compound \(5 (R^1 = \text{L-Ala-D-Glu})\), but not the dansylated compounds \(1 (R^1 = \text{OH})\) and \(3 (R^1 = \text{L-Ala})\), was able to stimulate Nod2 activity in the luciferase assay (Fig. 2C). Together, these assays demonstrate that our modified muramyl peptides are biologically active, that the additional moiety (Dns or Bio-Ahx) does not hamper their Nod-stimulating capacity, and that they activate Nod proteins with the same peptidic sequence requirements as the natural muramyl peptides.

We next investigated if dansylated or biotinylated muramyl peptides could be used for direct fluorescence or immunofluorescence, respectively, to follow their delivery and trafficking within host cells. HeLa cells grown on coverslips were first stimulated for 30 min with dansylated muramyl peptides. Since the dansyl group is intrinsically fluorescent (excitation \(\lambda = 336\) nm; emission \(\lambda = 531\) nm), direct visualization can be achieved using an epifluorescence microscope. Unfortunately, the fluorescence emitted by the muramyl peptides internalized into HeLa cells was under the detection limit (data not shown), probably due to the absence of an amplification step (typically provided by the sequential use of two antibodies).

We therefore repeated these studies using a biotinylated muramyl peptide, which has the advantage of allowing an amplification step through the use of fluorescent streptavidin-conjugated molecules interacting with the biotin group. HeLa cells were stimulated with compound \(7\) or Biot-transferrin as a control, followed by Streptavidin-Alexa488 together with Transferrin-Alexa568. Using this technique, we successfully detected Bio-Ahx-MurNAc-tripeptide inside HeLa cells and demonstrated that the molecule was colocalized with transferrin, a marker of early and recycling endosomes (Fig. 3), in agreement with previous reports showing
Figure 5.3. Internalization of biotinylated muramyl peptides in HeLa cells.

(A-C) HeLa cells grown on coverslips were first stimulated for 30 min with either medium (A), 1 μg/ml Bio-Ahx-MurNAc-L-Ala-γ-D-Glu-meso-A_{2}pm 7 (B) or 1 μg/ml Biot-Transferrin (C). Next, 2 μg/ml Streptavidin-Alexa488 (Invitrogen) were added together with 1 μg/ml Transferrin-Alexa568 (Invitrogen) for 30 min. Finally, cells were washed, fixed (4% paraformaldehyde, 15 min) and stained with DAPI to visualize nuclei. Images were taken using Zeiss Z-1 epifluorescence microscope with a 63× oil fluorescence objective and deconvolved using Volocity software (Quorum Technologies). Transferrin-Alexa568 was used as a positive control in our experiments. Arrows indicate biotinylated muramyl peptide-containing vesicles. Tfn, transferrin. Biot, biotin. MP, compound 7. Strep, streptavidin.
that muramyl peptides are internalized into mammalian cells through clathrin-mediated endocytosis (Lee et al., 2009; Marina-Garcia et al., 2009).

We previously demonstrated that, following internalization into endosomes, Nod1-activating muramyl peptides were likely processed by host hydrolases in the lumen of early endosomes in order to generate muramyl-free peptides that can be transported to the cytosol through the oligopeptide transporter SLC15A4, and possibly other transporters (Lee et al., 2009). Therefore, when using a biotinylated muramyl peptide to stimulate host cells, the molecule would be cleaved in the early endosome to generate Bio-Ahx-MurNAc and free peptide, with the former remaining in the lumen of the endosome and only the latter reaching the cytosol. We reasoned that this could be a major limitation of the use of biotinylated muramyl peptides for projects aiming to study the fate of muramyl peptides in the host cytosol (such as the identification of muramyl peptide-interacting proteins by immunoprecipitation). Consequently, it was important to demonstrate that a procedure could be used, in which delivery of the biotinylated muramyl peptide to cytosolic Nod proteins would occur independently from endocytosis.

To circumvent this potential limitation to the use of muramyl peptides derivatives in live cells because of endosomal processing, we stimulated HEK293T cells with biotinylated muramyl peptides using a modified procedure that we have used previously for unmodified muramyl peptides (Girardin et al., 2003a), in which cytosolic delivery is direct and does not require endocytic trafficking. In this experimental set-up, HEK293T cells were first transfected overnight with the Igκ-luci reporter plasmid, along with either pcDNA3, or expression vectors encoding for Nod1 or Nod2. The following day, biotinylated muramyl peptides were added together with digitonin, a plant-derived toxin that destabilizes and permeabilize host membranes, thus allowing direct delivery of molecules to the cytosol. Accordingly, cells were pulsed for 10 min in a permeabilization medium containing digitonin plus biotinylated muramyl peptides, before replacing this buffer with regular cell culture medium for another 6 h prior to cell lysis and luciferase measurement. Of note, we first verified in our assays that digitonin-mediated delivery of the muramyl peptides was independent from endosomal trafficking, by showing that activation of Nod1-dependent signaling in digitonin-permeabilized cells by MurNAc-L-Ala-γ-D-Glu-meso-A₂pm was insensitive to dynasore, a drug that efficiently inhibits clathrin-mediated endocytosis (data not shown).
Interestingly, we observed that these experimental conditions were also suitable for allowing delivery of biotinylated muramyl peptides to the cytosol, where compounds 7 and 6 could trigger activation of Nod1 and Nod2, respectively (Fig. 4). This demonstrates that the addition of the biotin-hydrazone arm to muramyl peptides does not affect the capacity of the molecules to access the cytosol through digitonin-mediated membrane permeabilization. Of note, in this experimental system, compound 7 was able to stimulate HEK293T cells endogenously (Fig. 4A), which likely explains why this compound also displayed activity in Nod2-overexpressing cells (see Fig. 4C). We did not observe such a capacity of biotinylated muramyl peptides to stimulate Nod pathways endogenously when trafficking through the endocytic machinery (see Fig. 2), even though similar concentrations of muramyl peptides were used, which suggests that digitonin-mediated delivery allows for a more efficient internalization of muramyl peptides than natural endocytosis. Together, these data demonstrate that biotinylated muramyl peptides can be delivered to the host cytosol where they efficiently trigger Nod activation. This digitonin-based procedure will therefore be useful to study biochemical aspects of the activation of host cells by muramyl peptides, such as the nature of the protein complexes interacting with these bacterial molecules. Indeed, an important open question in the field of Nod biology is to determine if Nod proteins directly interact with muramyl peptides, or if yet unknown adaptor molecules are required for detection and activation.

5.5 Conclusions & Future Directions

These experiments validate the approach of coupling muramyl peptides to the biotin probe. Using the biotin group as a bait, it can also be envisioned to use such derivatives to perform biochemical analyses of the proteins interacting with these bacterial molecules. Therefore, biotinylated muramyl peptides represent useful derivatives of Nod1/2 ligands, which will be critical for the understanding of how muramyl peptides activate the host innate immune system.
Figure 5.4. Enforced internalization of biotinylated muramyl peptides is sufficient to activate Nod1 and Nod2.

(A-C) HEK293T cells were transfected overnight with the reporter gene Igκ-luci to monitor NF-κB activation, together with the empty vector pcDNA3 (A) or either of the peptidoglycan sensors Nod1 (B) or Nod2 (C). The following day, cells were transiently permeabilized using digitonin, in the presence or absence of biotinylated muramyl peptides (2, Bio-Ahx-MurNAc; 9, Bio-Ahx-MurNAc-L-Ala-γ-D-Glu-meso-A2pm-D-Ala-D-Ala; 7, Bio-Ahx-MurNAc-L-Ala-γ-D-Glu-meso-A2pm; 6, Bio-Ahx-MurNAc-L-Ala-D-Glu), or with the positive controls Tri-A2pm (for Nod1, in (A-B)) or MDP (for Nod2, in (C)), as indicated, and luciferase activity was measured 4 h post-stimulation (B-D). All biotinylated muramyl peptides used were added at the final concentration of 50 nM, and positive controls were at 10 μg/ml. NS, non stimulated.
Chapter 6
The Role of Mouse Peptidoglycan Recognition Protein PGLYRP2 in the Innate Immune Response to *Salmonella enterica* serovar Typhimurium Infection in vivo

This chapter was published as


Contribution of Data

All experiments/analyses were performed by Jooeun Lee, unless noted otherwise;

Figure 5.4 and 5.5 – histological scoring of cecum samples was performed by Catherine Streutker. The subsequent analysis of the data was performed by Jooeun Lee.
6

6.1 Abstract

Peptidoglycan recognition proteins (PGRPs or PGLYRPs) are a family of innate pattern recognition molecules that bind bacterial peptidoglycan. While the key role of PGRPs in Drosophila innate immunity has been extensively studied, how the four mammalian PGLYRP proteins (PGLYRP1-4) contribute to host defense against bacterial pathogens in vivo remains poorly understood. PGLYRP1, PGLYRP3 and PGLYRP4 are directly bactericidal in vitro whereas PGLYRP2 is an N-acetylmuramyl-L-alanine amidase that cleaves peptidoglycan between the sugar backbone and the peptide stem. Because PGLYRP2 cleaves muramyl peptides detected by host peptidoglycan sensors Nod1 and Nod2, we speculated that PGLYRP2 may act as a modifier of Nod1/Nod2-dependent innate immune responses. We investigated the role of PGLYRP2 in Salmonella enterica serovar Typhimurium induced colitis, which is regulated by Nod1/2 through the induction of an early Th17 response. PGLYRP2 did not contribute to expression of Th17-associated cytokines, IL-22-dependent antimicrobial proteins, or inflammatory cytokines. However, we found that Pglyrp2-deficient mice displayed significantly enhanced inflammation in the cecum at 72h post-infection, reflected by increased polymorphonuclear leukocyte infiltration and goblet cell depletion. Pglyrp2 expression was also induced in the cecum of Salmonella-infected mice. Furthermore, expression of green fluorescent protein under control of Pglyrp2 promoter was increased in discrete populations, including intraepithelial lymphocytes, in response to infection. Lastly, Nod2−/− Pglyrp2−/− mice displayed a more severe early susceptibility to infection at 24h post-infection than Pglyrp2−/− mice, which correlated with increased PMN infiltration and submucosal edema. Thus, PGLYRP2 plays a protective role in vivo in the control of S. Typhimurium infection through a Nod1/2-independent mechanism.

6.2 Introduction

Innate immunity, the first line of defense against microorganisms, relies on pattern recognition molecules (PRMs), such as Toll-like receptors (TLRs) or nucleotide-binding and oligomerization domain (Nod)-like receptors (NLRs), which initiate protective responses against pathogens by detecting microbe-associated molecular patterns (MAMPs) (Fritz et al., 2006). In the intestine, mucosal defense against enteric pathogens critically depends on the expression of TLRs and
NLRs (Uematsu and Fujimoto, 2010), and these PRMs also contribute to the establishment of a homeostatic control of the intestinal microbiota (Magalhaes et al., 2007; Rakoff-Nahoum et al., 2004).

Peptidoglycan is an essential component of the cell wall of virtually all bacteria, and is sensed by a variety of PRMs in the mammalian host, including Nod1, Nod2 and peptidoglycan recognition proteins (PGRPs or PGLYRPs) (Girardin and Philpott, 2004; Royet and Dziarski, 2007). Nod1 and Nod2 are two well characterized members of the NLR family that detect peptidoglycan-derived muramyl peptides (Geddes et al., 2009). Specifically, Nod1 detects mesoDAP (diaminopimelic acid)-containing muramyl tripeptides found mostly in Gram-negative bacteria (Chamaillard et al., 2003; Girardin et al., 2003a) whereas Nod2 recognizes muramyl dipeptide (MDP), a peptidoglycan motif found in both Gram-negative and Gram-positive bacteria (Girardin et al., 2003b; Inohara et al., 2003). PGRPs, first identified in silkworm (Yoshida et al., 1996), are conserved from insects to mammals, and are characterized by their ability to bind peptidoglycan (Royet and Dziarski, 2007). In mammals, there are four PGRPs, namely PGLYRP1, PGLYRP2, PGLYRP3, and PGLYRP4 (initially named PGRP-S, -L, Iα, and Iβ, respectively). PGRPs are all capable of binding peptidoglycan (Royet and Dziarski, 2007); PGLYRP1, PGLYRP3, and PGLYRP4 are directly bactericidal (Kashyap et al., 2011; Lu et al., 2006; Tydell et al., 2006; Wang et al., 2007), but have no amidase activity (Kashyap et al., 2011; Lu et al., 2006; Wang et al., 2003), whereas PGLYRP2 is a N-acetylmuramyl-L-alanine amidase that hydrolyzes peptidoglycan between the sugar backbone and the peptide chain (Gelius et al., 2003; Wang et al., 2003).

PGLYRP2 is constitutively expressed in the liver where it is secreted into the blood (Xu et al., 2004; Zhang et al., 2005), and its expression is induced by bacteria and cytokines in the skin and in epithelial cells including those that line the intestinal tract (Uehara et al., 2005; Wang et al., 2005). Interestingly, based on the fact that PGLYRP2 cleaves muramyl peptides that are also detected by Nod1 and Nod2, it is possible that this PGRP protein could act as a modulator of Nod-dependent responses. In Drosophila, PGRP-LB also has amidase activity, which was shown to protect the host from excessive immune responses by reducing the biological activity of peptidoglycan (Paredes et al., 2011; Zaidman-Remy et al., 2006). However, it remains unclear whether the amidase activity of mammalian PGLYRP2 plays a similar anti-inflammatory scavenger function in vivo, in response to bacterial pathogens.
Despite the fact that PGRPs specifically bind bacterial peptidoglycan and that some of them display antibacterial activity in vitro (Lu et al., 2006; Tydell et al., 2002; Tydell et al., 2006; Wang et al., 2007), the in vivo role of these molecules in host defense against bacterial pathogens remains poorly understood in mammals. One study showed a requirement for PGLYRP1 in resistance to some infections in mice, as Pglyrp1-deficient mice showed increased susceptibility to systemic infection with *Bacillus subtilis* and *Micrococcus luteus*, but not with other Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*) (Dziarski et al., 2003). More recently, another study suggested a role of PGLYRP1 in host resistance against *Listeria monocytogenes* through the induction of TNF (Osanai et al., 2011). Similarly, using recombinant PGLYRP3 in wild-type mice, a bactericidal role of PGLYRP3 in preventing *S. aureus* lung infection in mice has also been suggested (Lu et al., 2006). In contrast, PGLYRP2 was suggested to be redundant for immunity in mice, on the basis of the fact that *Pglyrp2*-deficient mice had a normal response to intraperitoneal infection with Gram-positive (*S. aureus*) or Gram-negative (*E. coli*) bacteria (Xu et al., 2004). It is possible that the four mammalian PGRP proteins display some redundant functions in innate immunity, thus resulting in mild phenotypes for mice lacking any of these molecules. In support for this, in a dextran sulfate sodium (DSS)-induced colitis model, all four individual PGRP knockout mice displayed relatively similar increased sensitivity to DSS and altered immune responses (Saha et al., 2010). However, PGLYRP2 is unique among mammalian PGRP proteins in that it is the only mammalian protein known to have N-acetylmuramyl-L-alanine amidase activity against peptidoglycan, and this protein is one of the most abundantly secreted enzymes in the body fluids of mammals (Vanderwinkel et al., 1995). We therefore speculated that PGLYRP2 might have a unique role in host defense that had not been identified previously using in vitro assays or intraperitoneal infection models, such as a role in mucosal innate immune defense.

*Salmonella enterica* serovar Typhimurium (SL1344) causes acute colitis in humans while the same organism causes a systemic disease with little or no intestinal inflammation in mice (Hapfelmeier and Hardt, 2005). However, when mice are pre-treated with the antibiotic streptomycin prior to infection with *S. Typhimurium*, they develop an acute inflammatory response in the cecum (Hapfelmeier and Hardt, 2005). Recently, Nod1 and Nod2 have been shown to modulate inflammation in the streptomycin-treated mouse model of *Salmonella* colitis (Geddes et al., 2010). Given that PGLYRP2 recognizes and hydrolyzes peptidoglycan fragments
that are also detected by Nod1 and Nod2, and because mammalian PGLYRP2 is expressed in the intestine (Duerr et al., 2010; Saha et al., 2010), we hypothesized that PGLYRP2 may play an important role in modulating Nod1 and Nod2 dependent inflammation in the gut. Therefore, we investigated the role of PGLYRP2 in a murine model of *S. enterica* serovar Typhimurium colitis.

### 6.3 Materials and Methods

**Mice.** *Pglyrp2*-deficient (*Pglyrp2<sup>−/−</sup>*) mice were originally obtained from Dr. Richard Locksley and C57BL/6 (wild-type) mice were purchased from Charles River Laboratories (Wilmington, MA). All mice were bred and housed under specific-pathogen-free conditions in the animal facility of the Center for Cellular and Biomolecular Research, Toronto, Canada. All animal experiments were approved by the Animal Ethics Review Committee of the University of Toronto. In order to minimize variables affecting the outcome of this study, C57BL/6 WT mice were first crossed with fully backcrossed *Pglyrp2<sup>−/−</sup>* mice (Xu et al., 2004) to generate heterozygous F1 littermates and subsequently, these F1 mice were crossed again to generate F2 littermates. Genotyping F2 littermates was performed using the following primer sets: for WT; 5'-GGCTCTCTACTCCCACACAACC-3', 5'- GCAGCAATCCAAGCAGGATCC-3', and for *Pglyrp2*; 5'-GGCTCTCTACTCCCACACAACC-3', 5'-GCCGGACACGCTGAAC TTGTGG-3'. WT and *Pglyrp2<sup>−/−</sup>* mice generated from littermates were used in all our infection studies, and *Pglyrp2<sup>+</sup>/−* mice were used to study GFP+ PGLYRP2 expression by flow cytometry. *Nod1<sup>−/−</sup>* *Pglyrp2<sup>−/−</sup>* DKO and *Nod2<sup>−/−</sup>* *Pglyrp2<sup>−/−</sup>* DKO mice were generated by crossing either *Nod1<sup>−/−</sup>* (Millennium Pharmaceuticals) or *Nod2<sup>−/−</sup>* mice (Prof. Jean-Pierre Hugot, (Barreau et al., 2007)) with *Pglyrp2<sup>−/−</sup>* mice littermates generated from above. Genotyping was carried out using the following primer sets: for *Nod1*; 5'-CTTAGGCGATGACTCCCTCCTGTGC-3', 5'-GATCTTGCAGTATTAATGTGGGAG TGAC-3', 5'-CCATTGCAGGC TGCGCAA CTGTTG-3', and for *Nod2*; 5'-AACCGCATT ATTCGATGGGGC-3', 5'-GTCATTTC TGACCTCCTGACC-3', 5'-GCCTGCTCTCTTTA CTGAAGGCTC-3'.

**Bacterial infections.** 8 to 10 week-old WT and *Pglyrp2<sup>−/−</sup>* mice were fasted for 3h prior to oral administration of 20mg of streptomycin. Mice were again fasted for 3h after twenty-one hours following streptomycin treatment, and then infected via oral gavage with 5 x 10<sup>7</sup> CFU of SL1344, a streptomycin-resistant strain of *Salmonella enterica* serovar Typhimurium. Overnight
cultures of SL1344 were washed with phosphate-buffered saline (PBS), diluted to the desired CFU level based on optical density readings at 600nm and used for infections.

**Pathological scoring.** The distal half of cecum samples were collected for histology after mice were sacrificed, fixed in 10% formalin and then stained with hematoxylin and eosin (H/E) at the Toronto Center of Phenogenomics by standard histological staining procedures. H/E-stained cecum samples were then analyzed in blinded manner by a pathologist specializing in intestinal inflammation. The scoring system was based on a previous publication (Barthel et al., 2003) that was slightly modified to make the scoring of neutrophil (PMN) recruitment and goblet cell depletion more empirical (Geddes et al., 2010).

**Bacterial load quantification.** The spleen and cecal tissue samples were collected from infected mice and placed in PBS containing 1% Triton X-100 or PBS, respectively, and then homogenized using a rotor homogenizer. A small (10μl) cecal sample was further diluted in PBS containing 1% Triton X-100. Both splenic and cecal samples were then serially diluted in PBS and plated on MacConkey agar containing 50μg/ml streptomycin.

**Quantitative real-time PCR.** Cecum samples for qRT-PCR were collected and stored in RNALater (Sigma), then RNA was extracted with Qiagen RNasy Extraction kits. Genomic DNA was digested with Turbo DNase (Ambion) before reverse transcription to cDNA with SuperScript RTIII (Invitrogen). qRT-PCR was performed with SYBR Green (Applied Biosystems). The following primer sequences, which have been described elsewhere, were used in the current study: *Il17a*, forward, 5’-GCTCCAGAAGGCCCTCAGA-3’, reverse, 5’-CTTTCCTCCGCATTGACA-3’; *Il22*, forward, 5’- TCCAGAGTAGTCAGTGCTAAA-3’, reverse, 5’-AGAACGTCTTCCAGGGTGAA-3’; housekeeping gene *Rpl19*, forward, 5’-GCATCCTCATGGAGCACAT-3’, reverse, 5’-CTGTGTCAGCTGCTTCTCTT-3’; *RegIIIy*, forward, 5’-ATGGCTCCTATTGCTATGCC-3’, reverse, 5’-GATGTCTCTAGGGGCCTC-3’; *Lcn2*, forward, 5’-ACATTTGTCCAGGCTCCAG GGC-3’, reverse, 5’-CATGGCGAACTGTTGTAGTCCAG-3’; *Il1β*, forward 5’-TTGACGGACCCAAAAGATG-3’, reverse 5’- AGAAAGGTGC TCATGTCTCAT-3’, *KC*, forward 5’-ACTGCACCCAAAACGAAGTC-3’, reverse 5’-CAAGGAGCTTTCA GGGTCAAA-3’; *Pglyrp2*, forward, 5’-ACCAGGATGTGCAGCAGTGGAT-3’ reverse, 5’-
AGTGACCCAGTGTAGTTGCCCA-3’. Values were calculated using the ΔCt method and were normalized to the housekeeping gene Rpl19.

**Lamina propria lymphocyte and intraepithelial leukocyte isolation.** Cecal IELs and LPLs were prepared using a previously established protocol (Geddes et al., 2011). Briefly, cecal tissues were extracted, washed and cut into 1 to 2 cm segments which were incubated three times (37°C, 10 min, shaking) in stripping buffer (PBS, 1% FBS, 5mM EDTA, 1mM DTT). After each incubation, the buffer was filtered through a 100-µm cell strainer and then allowed to settle. Supernatants (IELs) were then collected, washed twice in DMEM (20% FBS) and passed through a 40-µm cell strainer. For LPLs extraction, the tissue segments following stripping were minced and put in digestion buffer (DMEM, 20% FBS, 2mg/ml collagenase D (Roche), 20µg/ml DNase I (Sigma)) for two 30-min incubations with shaking at 37°C. Digested material was passed through a 100-µm cell strainer, and the cells were collected by centrifugation (5 min, 1200 rpm), washed twice in DMEM and then passed through a 40-µm cell strainer to obtain LPLs.

**Flow cytometry.** Dead cells were stained with Live/Dead Aqua (Invitrogen), and then LPLs and IELs were stained for surface antigens, CD8, CD4, TCRβ, TCRγδ, CD11c, CD11b, MHCII, CD19, NK1.1, and Gr1 and analyzed for GFP expression. All flow cytometric analyses were performed using a LSR II (BD Bioscience) flow cytometer, and analyzed with FlowJo software (TreeStar).

**Statistical analyses.** The results are given as means ± standard error of mean. Student’s t tests were performed using Graphpad (Prism), and P values < 0.05 using a 95% confidence interval were considered significant.

### 6.4 Results

**PGLYRP2 is constitutively expressed in some subsets of IEL and LPL in the cecum.** Pglyrp2 mRNA expression in the total intestinal tissues has been previously described (Saha et al., 2010). In addition, Pglyrp2 expression was recently characterized in small intestinal intraepithelial leukocytes (IELs), with the majority of its expression in T lymphocytes (Duerr et al., 2010). Here, we focused on the cecum, since this portion of the intestine was recently found
to be critical for Nod1/2-dependent control of enteric bacterial pathogens (Geddes et al., 2010; Geddes et al., 2011). The \textit{Pglyr2}-deficient (\textit{Pglyr2}^{−/−}) mice used in this study express green fluorescent protein (GFP) under the control of the \textit{Pglyr2} promoter (Xu et al., 2004), and therefore, we used flow cytometry to monitor PGLYRP2 expression in cecal IELs and lamina propria lymphocytes (LPLs) by tracking GFP expression in \textit{Pglyr2}^{−/−} mice (to avoid a possible effect on immune cell populations that may occur due to loss of PGLYRP2 expression in \textit{Pglyr2}^{−/−} mice). At steady-state, we confirmed the previously reported high expression of PGLYRP2 in lymphocytes from liver and spleen (data not shown) (Duerr et al., 2010). We also detected strong expression of GFP cells in the IELs of the cecum, mainly in CD4+TCRβ+ (49.7%), CD8+TCRβ+ (73.5%) lymphocytes, natural killer cells (NK1.1+TCRβ−, 76.2%) and natural killer T cell (NK1.1+TCRβ+, 71.4%) populations (Fig. 5.1A, Table 5.1). In contrast to a previous report where PGLYRP2 expression was found to be restricted to T lymphocytes from intestinal IELs (Duerr et al., 2010), we observed that dendritic cells (CD11c+MHCII+, 49.7%), and inflammatory monocytes (CD11b+CD11c+, 23.5%) from the cecal IEL compartment also exhibited a varying degree of GFP+ PGLYRP2 expression (Fig. 5.1A, Table 5.1). Conversely, minimal GFP+ PGLYRP2 expression was observed on CD19+ (3.9%) subset from IELs, indicating absence of reporter gene expression by B lymphocytes. Moreover, no significant GFP+ PGLYRP2 expression was noted in CD11b+CD11c− (granulocytes/macrophage/neutrophil) cells (data not shown), and further staining with Gr1 marker indicated that neither neutrophils (CD11b+CD11c−Gr1+, 1.7%) nor macrophages (CD11b+CD11c−Gr1−, <1.0%) expressed significant level of GFP+ PGLYRP2 at baseline (Table 5.1). Similar results were obtained for cecal LPLs, except in NK1.1+TCRβ+ (1.95%), CD11c+MHCII+ (<1.0%), and CD11b+CD11c+ (<1.0%) where no significant GFP+ expression was detected (Fig. 5.1B, Table 5.1). Overall, PGLYRP2 is constitutively expressed by various leukocytes from the cecum.
Figure 6.1. Analysis of PGLYRP2 expression in intestinal cellular subsets.

Intraepithelial leukocytes (IEL) and lamina propria lymphocytes (LPL) were prepared from cecal samples of wild-type and Pglyrp2+/- mice, stained with various surface markers (CD4+, TCRβ+, CD8+, CD19+, NK1.1+, CD11c+) and analyzed by flow cytometry for GFP expression. Histograms show expression of GFP+ cells on gated subsets (CD4+TCRβ+, CD8+TCRβ+, CD19+, NK1.1+TCRβ+, NK1.1+TCRβ−, CD11c+MHCII+) from IEL (A) and LPL (B). The percentage of GFP-expressing cells in Pglyrp2+/- mice was determined using wild-type cells as a baseline (shaded area). One representative of three experiments is shown, three mice were pooled for each group.
Table 6.1. Detailed characterization of the cecal leukocyte population expressing reporter gene carrying the GFP+ under the control of the Pglyrp2 promoter at steady-state.

The data shown is the average of three independent experiments as mean ± s.e.m.
**PGLYRP2 expression is increased in the cecum during late inflammatory responses against SL1344 infection.**

PGLYRP2 expression is induced in intestinal epithelial cells and fibroblasts by bacteria and cytokines (Ma et al., 2010; Uehara et al., 2005; Wang et al., 2005). Thus, we aimed to analyze whether PGLYRP2 expression changed following oral infection with *S. enterica* serovar Typhimurium SL1344. To this end, C57BL/6 wild-type (WT) mice were treated with streptomycin and infected with $5 \times 10^7$ CFU of the streptomycin-resistant *Salmonella* strain, SL1344. We performed quantitative real-time PCR (qRT-PCR) on the cecum samples from the WT mice following SL1344 infection to determine Pglyrp2 mRNA expression. Although no difference in expression was observed at 24h, the level of *Pglyrp2* showed approximately 1.7- and 2.3-fold increase at 48h ($p=0.02$) and 72h ($p=0.03$) post-infection, respectively, compared to the uninfected controls (Fig. 5.2A). In order to determine which cell types contributed to the increase in *Pglyrp2* expression, we analyzed IELs and LPLs from *Pglyrp2*+/− ceca infected with *Salmonella* at 48h by flow cytometry. The analysis of GFP expression in CD8+TCRβ+ cells from the IEL compartment showed a significantly increased expression of PGLYRP2 ($p=0.04$) following infection (Fig. 5.2B). A similar increase in expression ($p=0.05$) was noted in CD8+TCRγδ+ cells, although this corresponded to a minor IEL population (Fig. 5.2B). On the other hand, no significant difference in the GFP+/PGLYRP2 expression was observed in cells from LPL compartment (Fig. 5.2C). It appears that IEL CD8+TCRβ+ lymphocytes, the most abundant subset found among cecal leukocyte populations, also displayed the most prominent increase in the expression of PGLYRP2 following *Salmonella* infection. Together, these results establish that PGLYRP2 is readily induced in specific immune cell subsets of the intestinal mucosa, in response to enteric infection with *Salmonella*, thus suggesting a role for this peptidoglycan-interacting molecule in host defense in vivo.
Figure 6.2. *Pglyrp2* expression is increased in the cecum following *Salmonella* infection.

qRT-PCR was used to measure the expression of *Pglyrp2* in the cecum from uninfected and infected WT mice at 24h, 48h and 72h (A). The expression is normalized to the housekeeping gene *Rpl19* (six to eight mice per group per experiment. n=3. *P<0.05). Bar graph (A) shows average fold change over uninfected controls (n=3). The average relative number of GFP+ cells in different populations (CD4+TCRβ+, CD8+TCRβ+, CD8+TCRγδ+, NK1.1+TCRβ−, NK1.1+TCRβ+, CD19+, CD11c+MHCII+, Gr1+CD11b+) in cecal IELs (B) and LPLs (C) from *Pglyrp2*+/− mice uninfected or 48h after infection with SL1344, as quantified by flow cytometry analysis (n=3, three mice pooled per group, *P<0.05).
**PGLYRP2 does not play a role in early Nod1/2-dependent Th17 response to *Salmonella* infection.** As shown above, PGLYRP2 is expressed in immune cell population of the intestinal mucosa, and *Pglyrp2* mRNA levels are significantly elevated in this tissue in response to *Salmonella* infection (Figs. 5.1 and 5.2). We then speculated that PGLYRP2, through its capacity to bind or to hydrolyze peptidoglycan, may serve as a modulator of Nod1/2-dependent host defense in response to *Salmonella* infection. Therefore, we investigated the putative role of PGLYRP2 in Th17-dependent inflammation in *Salmonella* colitis, because this arm of the host response to *Salmonella* was shown to depend on Nod1 and Nod2 (Geddes et al., 2010; Geddes et al., 2011). To this end, WT and *Pglyrp2*−/− mice were treated with streptomycin and infected with 5 x 10⁷ CFU of SL1344. Mice were sacrificed at 24h, 48h or 72h following infection, and then their ceca were homogenized for analysis. qRT-PCR analysis of *Il17a*, and *Il22* from WT and *Pglyrp2*−/− cecum revealed no significant changes in their expression over the period of infection (Fig. 5.3A). Moreover, the expression of *Lcn2* (encoding lipocalin 2) and *RegIIIγ* (encoding regenerating islet-derived IIIγ), the antimicrobial proteins important for IL-22-dependent mucosal defense against enteric bacteria (Raffatellu et al., 2009), did not change significantly in the cecum of *Pglyrp2*−/− mice compared to that of WT mice (Fig. 5.3B).

Nod1 and Nod2 regulate the levels of key inflammatory cytokines such as the keratinocyte-derived chemokine (KC) and IL-1β during *Salmonella* colitis (Geddes et al., 2010). Therefore, we assessed the impact of PGLYRP2 on the expression of the cytokines *KC* and *Il1β* during infection. qRT-PCR analysis from WT and *Pglyrp2*−/− cecal samples showed no significant differences in the mRNA level of these inflammatory cytokines (Fig. 5.3C), suggesting that, unlike Nod1 and Nod2, PGLYRP2 expression does not have an impact on the level of these inflammatory cytokines during *Salmonella* infection. Overall, these findings indicate that PGLYRP2 does not modulate Nod1-, and Nod2-dependent early Th17 inflammatory responses during *Salmonella* colitis.
Figure 6.3. PGLYRP2 does not modulate Nod-dependent early Th17 responses or inflammatory cytokine responses to *Salmonella* infection.

qRT-PCR was used to measure the mRNA level of (A) *Il17a, Il22*, (B) *Lcn2, RegIIIγ*, and (C) *KC, Il1β* in cecal samples from streptomycin-treated WT and *Pglyrp2*−/− mice infected with 5 x 10^7 CFU of SL1344 for 24 to 72h. Line graphs show the average expression levels of *Il17a* and *Il22* in infected samples over time (A). Bar graphs depict the average expression levels of antimicrobial peptides, *Lcn2, RegIIIγ* (B) and inflammatory cytokines, *KC*, and *Il1β* (C) in both uninfected and infected samples at 72h. The expression is normalized to the housekeeping gene *Rpl19* (six to eight mice per group per experiment. One representative of three experiments is shown). Error bars represent 1 standard error of the mean (s.e.m). NS: not significant.
**Pglyrp2-deficient mice have increased inflammation in the cecum following Salmonella infection.** Although PGLYRP2 does not appear to affect the Nod1/2-dependent Th17 inflammatory responses, this does not exclude a possibility that PGLYRP2 may still play a role independent of Nod1 and Nod2 in Salmonella colitis. To test this hypothesis, we stained cecum samples from uninfected and infected WT and Pglyrp2−/− mice and performed histological analysis. The analyzed features included polymorphonuclear leukocyte (PMN) accumulation, goblet cell depletion, edema, and epithelial erosion, which were blindly scored using a previously established scoring system (see Materials and Methods) (Fig. 5.4A). Although infected Pglyrp2−/− mice displayed no change in cecal inflammation compared to that of infected wild-type mice at early stages of infection (24-48h) (Fig. 5.4A-B), we noticed that the level of infiltrating PMNs was significantly higher in Pglyrp2−/− mice than WT mice at 48h post-infection (p=0.04) (Fig. 5.4C). Moreover, goblet cell depletion was also significantly elevated at 48h post-infection in Pglyrp2−/− mice, relative to WT mice (p= 0.01) (Fig. 5.4D). Despite the absence of significant differences at early time points of infection, we observed at 72h post-infection a marked increase in cecal inflammation in Pglyrp2−/− mice (p= 0.02) (Fig. 5.4A-B). This was reflected by significantly enhanced PMN recruitment, and goblet cell depletion in infected Pglyrp2−/− mice, compared to WT mice (p=0.03, and 0.003, respectively) (Fig. 5.4C-D). It must be noted that this increase in pathology did not follow the pathology seen in Nod1−/Nod2− double knockout (DKO) mice, where inflammation is significantly reduced following infection (Geddes et al., 2010). Finally, spleens and cecal tissue samples were examined from the same infected mice as those used for histological analysis to determine bacterial colonization and spread. Although Pglyrp2−/− mice had levels of colonization similar to those of WT mice in both spleen and cecum samples, a trend toward increased bacterial load (p=0.07) was observed in Pglyrp2−/− cecal tissues at 72h post-infection, compared to WT cecal tissues (Fig. 5.4E-F). Thus, our findings indicate that PGLYRP2 plays a protective role during the late stage of Salmonella colitis, and that this role is independent from Nod1- and Nod2-induced control of inflammation.
Figure 6.4. *Pglyrp2*−/− mice have increased inflammation following SL1344 infection.

Streptomycin-treated WT or *Pglyrp2*−/− (KO) mice were either PBS-treated (uninfected) or infected with 5 x 10⁷ CFU of SL1344 for 24 to 72h, then their ceca were examined for histological changes and bacterial loads. For histological changes, the average pathological scores for each analyzed feature (edema, neutrophil recruitment, goblet cell depletion, and epithelial erosion) for all mice from each group was calculated (A) as well as the average total sum of the pathological scores (B). The line graphs depict the average numbers of PMNs (C) and goblet cells (D) observed per microscopic field from infected samples over the period of infection. For bacterial loads, CFU counts were determined on cecal tissue (E) and spleen (F) samples from infected mice (n=3, six to eight mice per group per experiment). Error bars represent 1 s.e.m. Significant: *, P < 0.05; **, P < 0.01.
Cecal inflammation is greatly exacerbated in $\text{Nod2}^{-/-} \text{Pglyrp2}^{-/-}$ DKO mice following 24h post-infection with $\text{Salmonella SL1344}$. Next, we sought to investigate whether PGLYRP2 differentially influence Nod1 or Nod2 response to bacterial peptidoglycan in vivo. In order to carry out the investigation, we first crossed our $\text{Pglyrp2}^{-/-}$ mice with either $\text{Nod1}^{-/-}$ or $\text{Nod2}^{-/-}$ mice to generate the double knock-outs (DKO) of $\text{Nod1}^{-/-}\text{Pglyrp2}^{-/-}$ and $\text{Nod2}^{-/-}\text{Pglyrp2}^{-/-}$. We then performed histological analysis on the cecum samples from uninfected and infected WT, $\text{Pglyrp2}^{-/-}$, $\text{Nod1}^{-/-}\text{Pglyrp2}^{-/-}$ DKO and $\text{Nod2}^{-/-}\text{Pglyrp2}^{-/-}$ DKO mice at 24h. The individual pathological features were scored (Fig. 5.5A) and were subsequently combined to calculate the average pathological scores (Fig. 5.5B). Infected $\text{Nod1}^{-/-}\text{Pglyrp2}^{-/-}$ DKO mice displayed no significant change ($p=0.12$) in cecal inflammation compared to that of infected WT or $\text{Pglyrp2}^{-/-}$ mice at 24h (Fig. 5.5B). Interestingly, however, we observed that the level of cecal inflammation was greatly elevated ($p=0.005$) in $\text{Nod2}^{-/-}\text{Pglyrp2}^{-/-}$ DKO mice at 24h p.i. with $\text{Salmonella}$ (Fig. 5.5B). This increase was attributed to significantly higher level of infiltrating PMNs ($p=0.003$), as well as increased percentage of submucosal edema ($p=0.005$) in the cecal tissue of $\text{Nod2}^{-/-}\text{Pglyrp2}^{-/-}$ DKO mice compared to that of the WT mice (Fig. 5.5C-D). In contrast, goblet cell depletion did not seem to contribute to this enhanced phenotype as no difference was observed in the levels from the cecum of either WT or $\text{Nod2}^{-/-}\text{Pglyrp2}^{-/-}$ DKO mice (data not shown). Finally, both $\text{Nod1}^{-/-}\text{Pglyrp2}^{-/-}$ DKO and $\text{Nod2}^{-/-}\text{Pglyrp2}^{-/-}$ DKO mice had similar levels of colonization compared to those of WT mice in both spleen and cecum samples at 24h post-infection (Fig. 5.5E-F). In conclusion, $\text{Nod2}^{-/-}\text{Pglyrp2}^{-/-}$ DKO mice seem to have exacerbated inflammatory response to $\text{Salmonella}$ infection than $\text{Nod1}^{-/-}\text{Pglyrp2}^{-/-}$ DKO mice, suggesting that PGLYRP2 may differentially regulate the response by Nod1 and Nod2 to bacterial peptidoglycan, and that Nod2 signaling is likely intact and confers early protection against Salmonella infection in $\text{Pglyrp2}^{-/-}$ mice.
Figure 6.5. *Nod2<sup>−/−</sup>Pglyrp2<sup>−/−</sup>* mice have significantly increased inflammation at 24h following SL1344 infection.

Streptomycin-treated WT, *Pglyrp2<sup>−/−</sup>* (KO), *Nod1<sup>−/−</sup>Pglyrp2<sup>−/−</sup>* (PG1), *Nod2<sup>−/−</sup>Pglyrp2<sup>−/−</sup>* (PG2) mice were either PBS-treated (uninfected) or infected with 5 x 10<sup>7</sup> CFU of SL1344 for 24h, then their ceca were examined for histological changes and bacterial loads. For histological changes, the average pathological scores for each analyzed feature (edema, neutrophil recruitment, goblet cell depletion, and epithelial erosion) for all mice from each group was calculated (A) as well as the average total sum of the pathological scores (B). The line graphs depict the average numbers of PMNs (C) and the percentage of submucosal edema (D) observed per microscopic field from infected samples over the period of infection. For bacterial loads, CFU counts were determined on cecal tissue (E) and spleen (F) samples from infected mice (n=3, six to eight mice per group per experiment). Error bars represent 1 s.e.m. Significant: *, P < 0.05; **, P < 0.01; NS, non-significant.
6.5 Discussion

In the present study, we investigated the role of PGLYRP2 in *Salmonella enterica* serovar Typhimurium induced colitis. We found that *Pglyrp2* expression was increased in the cecum of *Salmonella*-infected mice and, by flow cytometry, we observed that expression of GFP was increased in discrete populations, including cecal lymphocytes in response to infection. Our results also demonstrated that PGLYRP2 did not contribute to the expression of Th17-associated cytokines, IL-22-dependent antimicrobial proteins, or inflammatory cytokines. However, *Pglyrp2*-deficient mice displayed significantly enhanced inflammation in the cecum at 72h post-infection, reflected by increased PMN infiltration and goblet cell depletion, thus showing for the first time a role for PGLYRP2 in host defense against an enteric bacterial pathogen in vivo. Moreover, *Nod2*<sup>−/−</sup>*Pglyrp2*<sup>−/−</sup> DKO mice displayed highly elevated level of inflammation at an earlier time point (24h p.i. infection) compared to its counterparts, WT, *Pglyrp2*<sup>−/−</sup>, and *Nod1*<sup>−/−</sup>*Pglyrp2*<sup>−/−</sup> DKO mice, suggesting intricate and complex relationships among these PRMs.

The processing and degradation of peptidoglycan by host enzymes such as PGLYRP2 and lysozyme can indirectly influence bacterial sensing by pattern-recognition receptors, such as Nod1 and Nod2. While lysozyme cleaves the sugar chain between GlcNAc (N-acetylglucosamine) and MurNAc (N-acetylmuramic acid), PGLYRP2 specifically hydrolyzes the lactyl bond between MurNAc and L-Ala, generating free peptide fragments, nonetheless still recognized by Nod1 (such as the tripeptide L-Ala-D-Glu-mesoDAP or the tetrapeptide L-Ala-D-Glu-mesoDAP-D-Ala) (Girardin et al., 2003a; Magalhaes et al., 2005). Similarly, PGLYRP2 was shown to hydrolyze the lysine-containing muramyl tripeptide MurNAc-L-Ala-D-Glu-Lys into L-Ala-D-Glu-Lys (Wang et al., 2003), which can no longer be recognized by Nod2 (Girardin et al., 2003b). Interestingly, the minimum peptidoglycan fragments hydrolyzed by PGLYRP2 are muramyl tripeptides (containing either *meso*DAP or Lys), and therefore, the typical Nod2 ligand MDP is not cleaved by PGLYRP2 (Wang et al., 2003), suggesting that MDP could remain biologically active to stimulate Nod2 regardless of the presence or absence of PGLYRP2. For this reason, it is likely that the hydrolytic activity of PGLYRP2 is not sufficient to functionally degrade Nod1/2-specific peptidoglycan fragments in vivo, although this has not been directly tested. In particular, it remains possible that, although MurNAc-L-Ala-D-Glu-*meso*DAP and L-
Ala-D-Glu-\textit{meso}DAP can activate Nod1 with similar capacity, the presence of the sugar moiety could modify cellular uptake, as we recently suggested (Lee et al., 2009). It is also possible that PGLYRP2 could differentially affect Nod2-dependent responses to bacteria, depending on the relative proportions of dipeptide versus lys-containing tripeptides in their peptidoglycan, a ratio that varies from one bacterial species to another. Nevertheless, these considerations strongly suggest that the amidase activity of mammalian PGLYRP2 may not be responsible for promoting a global scavenger function aiming to dampen immune responses dependent on peptidoglycan. This contrasts with the role assigned to \textit{Drosophila} amidase PGRPs, such as PGRP-LB. Indeed, in \textit{Drosophila}, detection of peptidoglycan and induction of innate immune responses to bacteria by effector PGRPs (such as PGRP-LC or PGRP-SA) require that peptidoglycan fragments contain both sugar and peptide moieties (Kaneko et al., 2004; Stenbak et al., 2004).

In our \textit{Salmonella} infection model, it appears that PGLYRP2 may trigger host defense and innate immune responses independently of its amidase activity, especially given that our histology data on infected mice did not follow the pathology observed in Nod1\(^{-/-}\)/Nod2\(^{-/-}\) double knockout mice where inflammation is significantly reduced during infection (Geddes et al.). Supporting this argument, an immune-modulatory role for PGLYRP2 irrespective of its amidase function has been recently shown in the arthritis inflammation model (Saha et al., 2009). In this study, the authors showed that both Nod2 and PGLYRP2 were required for the induction of peptidoglycan-induced arthritis, and that Nod2 was acting upstream of PGLYRP2 to induce its expression. Indeed, our data support the notion that Nod proteins and PGLYRP2 may be working together to promote protection in our colitis model. In our study, we observed a significant increase in cecal inflammation in Nod2\(^{-/-}\) Pglyrp2\(^{-/-}\) DKO mice, but not Nod1\(^{-/-}\) Pglyrp2\(^{-/-}\) DKO mice, at an early stage (24h) of \textit{Salmonella} infection, compared to that of WT or Pglyrp2\(^{+/+}\) mice. Our data thus suggest that both Nod2 and PGLYRP2 may be working in concert as part of a regulatory pathway where contribution of Nod1 is minimal, to confer protection during \textit{Salmonella} infection. These results are also in agreement with the previous biochemical data mentioned above, which demonstrated that PGLYPR2 was unable to degrade MDP, the muramyl peptide agonist of Nod2 (Wang et al., 2003).

In summary, we have demonstrated a protective role for PGLYRP2 during \textit{Salmonella} infection in vivo, and this represents the first indication that this molecule contributes to host defense
against bacterial pathogens at mucosal surfaces. Although it is clear that PGLYRP2 plays a protective role in down-regulating inflammation in *Salmonella*-induced colitis, whether this effect is mediated by amidase-dependent processing of peptidoglycan fragments and affecting Nod1/2-driven host responses is questionable, given that PGLYRP2 did not seem to modulate Nod1/2-dependent, early Th17 responses to *Salmonella* infection. Further research is required to delineate the mechanisms by which PGLRP2 confers protection in the intestine during bacterial infection.
Chapter 7
Discussion

7

7.1 Thesis summary

The central theme of this thesis has been the study of the innate immune molecules that recognize bacterial peptidoglycan (Figure 7.1). In particular, the first three data chapters (Chapter 3-5) focused mainly on Nod1 and Nod2, the intracellular innate immune receptors crucial for recognizing peptidoglycan, with respect to the downstream signaling molecule of the receptors, and to the entry mechanism of their respective ligands. First, the contribution of Rip2, the adaptor protein for Nod1- and Nod2-dependent signaling, in innate and adaptive immunity was investigated in vivo using Rip2-deficient mice (Chapter 3). I demonstrated that Rip2 was required and critical for triggering Nod1- and Nod2-driven induction of innate and adaptive immunity in vivo. Next, I investigated how monomeric peptidoglycan fragments can enter into epithelial cells to be detected by Nod1 and Nod2 (Chapter 4). Using biochemical approaches and molecular biology techniques in vitro, I determined that the entry of these Nod1 and Nod2 ligands was mediated through clathrin-dependent endocytosis, and the internalization of the ligands was dependent on pH. More importantly, I identified a role for SLC15A4, an oligopeptide transporter expressed in early endosomes, in Nod1-dependent signaling. While investigating the role of SLC15A4, a surprising observation (Fig 4.4D) was made which alluded to a possible role for the host hydrolytic enzyme, PGLYRP2, in cleaving Nod1 ligands prior to transport into cytosol. Thus, the last chapter of this thesis was dedicated to further investigating the role of the peptidoglycan recognition molecule, PGLYRP2, in induction of innate immune response to Salmonella enterica serovar Typhimurium infection (Chapter 6). Initially, I hypothesized that PGLYRP2 can indirectly influence bacterial peptidoglycan sensing by Nod1 and Nod2, since PGLYRP2 is known to cleave Nod1 and Nod2 ligands. Surprisingly, I found that PGLYRP2 did not modulate Nod1- and Nod2-dependent inflammatory responses during Salmonella colitis in vivo. Rather, PGLYRP2 displayed a protective role in the host defense against S. Typhimurium, and this role appeared to be independent of its amidase activity. Together, this thesis has provided a better understanding to some important questions in Nod biology, and formulated many new questions for future investigation.
The central theme of my thesis was to address some of the important questions surrounding the muramyl peptides. Since the discovery of Nod1 and Nod2, it has been a critical issue to understand how these muramyl peptides enter the cells to activate Nod1 and Nod2. Using an *in vitro* assay, I showed a mechanism of entry in epithelial cells, and provided the first evidence that SLC15A4 is important in trafficking Nod1 ligands. Further, my work on Rip2 nicely settled the age-old debate, confirming that Rip2 is fully required for all Nod-dependent signaling *in vivo* triggered by these ligands. Lastly, despite the fact that PGLYRP2 is one of the most abundantly secreted enzymes in body fluids of mammals, and it has the ability to process Nod ligands, not much had been known as to what the function of this enzyme might be. My work demonstrated a protective role of PGLYRP2 *in vivo*, which had not been previously identified in bacterial colitis model.

Figure 7.1. Summary of my thesis.

The central theme of my thesis was to address some of the important questions surrounding the muramyl peptides. Since the discovery of Nod1 and Nod2, it has been a critical issue to understand how these muramyl peptides enter the cells to activate Nod1 and Nod2. Using an *in vitro* assay, I showed a mechanism of entry in epithelial cells, and provided the first evidence that SLC15A4 is important in trafficking Nod1 ligands. Further, my work on Rip2 nicely settled the age-old debate, confirming that Rip2 is fully required for all Nod-dependent signaling *in vivo* triggered by these ligands. Lastly, despite the fact that PGLYRP2 is one of the most abundantly secreted enzymes in body fluids of mammals, and it has the ability to process Nod ligands, not much had been known as to what the function of this enzyme might be. My work demonstrated a protective role of PGLYRP2 *in vivo*, which had not been previously identified in bacterial colitis model.
7.2 Revisiting the entry mechanism of Nod1 ligands

One of the central dogmas in Nod biology was how free-floating extracellular peptidoglycan fragments get access to the cytosol of a cell where Nod1 or Nod2 receptors reside. My work contributed to finding one of the entry mechanisms of muramyl peptides into epithelial cells, introducing SLC15A4 as an emerging transporter involved in trafficking of the ligands inside the epithelial cells. Since then, other publications came forth in support of SLC15A4 playing a role in trafficking of ligands, especially in APCs such as dendritic cells. For instance, SLC15A4 is required for endosomal TLR7 and TLR9 signaling in plasmacytoid dendritic cells (Blasius et al., 2010). The authors hypothesize that SLC15A4 may play a role in transporting a critical component in and out of the endosome, and/or maintaining the appropriate pH of the endosomal compartment as endosomal acidification is necessary for optimal TLR7 and TLR9 activation. Similarly, studies in Slc15a4−/− dendritic cells suggest that SLC15A4 contributes to TLR9 signaling by regulating endosomal histidine levels (Sasawatari et al., 2011). With regards to Nod1 ligands, SLC15A4 was also shown to function as a transporter of TriDAP in vivo as Slc15a4−/− mice demonstrated a severe defect in Nod1-dependent cytokine production (Sasawatari et al., 2011).

Intriguingly, some of the SLC subfamilies that could be potentially implicated in the transport of peptidoglycan-derived peptides have also been associated with the pathophysiology of IBD. It has been reported that SLC22A5 gene is important susceptibility genes in IBD, and that genetic polymorphisms within this region contribute to the inflammatory phenotype (Cucchiara et al., 2007; Waller et al., 2006). Among SLC15 transporters, polymorphisms in genes encoding SLC15A1 have been linked to IBD (Zucchelli et al., 2009). SLC15A1 was also reported to be involved in the suppression of inflammation in mouse models of colitis because of uptake of the therapeutic tripeptide KPV (Dalmasso et al., 2008). On the contrary, no genetic evidence linking SLC15A4 to IBD exist yet, although SLC15A4 has been identified as one of susceptibility genes associated with the autoimmune disease, systemic lupus erythematosus (SLE) (Han et al., 2009; He et al., 2010). Interestingly, our collaborators demonstrated that SLC15A4 mRNA expression level was increased in inflamed colon of IBD patients compared to control subjects. Further, it was recently reported that SLC15A4 deficiency ameliorated susceptibility to DSS colitis in mice (Sasawatari et al., 2011). Together, these findings suggest that SLC15A4 may have a role in the pathophysiology of IBD. In a global view, these findings and the presence of genes such as
SLC22A5, and SLC15A1 in IBD-risk loci suggest that solute carriers may help to maintain intestinal homeostasis by transducing signals from microbial ligands, which can in turn have immune-modulatory effects on the host.

Future directions: Following my work that showed SLC15A4 is involved in transporting Nod1 ligands in epithelial cells, others have found the expression of SLC15A4 in immune cells such as B cells and dendritic cells (Sasawatari et al., 2011). It would thus be interesting to determine if SLC15A4 is functional in these cell types that can potentially be in contact with peptidoglycan, and investigate whether this protein plays a role in transporting Nod1 ligands into these cells. Further, in vivo processing/transporting capability of the ligands could be examined by injecting various Nod1 ligands (M-Tri-DAP, iE-DAP, TCT, FK156) into SLC15A4-deficient mice, and measuring the inflammatory cytokine responses, compared to WT mice. Lastly, it would be interesting to investigate how the specific internalization of Nod1 ligands by SLC14A4 might contribute to the detection of harmful pathogens at the epithelial lining of the mucosa nearby. To study this, a conditional knockout of SLC15A4 in the intestinal epithelial cells should be generated, using floxed mice crossed with transgenic animals expressing the Cre recombinase under control of the villin promoter, a gene specifically expressed in the gut epithelium. Together, these studies are of critical importance in further understanding the role of SLC15A4 in innate immunity.

7.3 PGLYRP2 : unpublished observations & future directions

Antimicrobial role of PGLYRP2

The study featured in Chapter 6 of this thesis characterized a new role of PGLYRP2, which has not been identified previously in bacterial colitis models. Although I have not been able to identify the exact mechanism by which PGLYRP2 confers protection in bacterial colitis, evidence seems to indicate that the amidase activity of PGLYRP2 may not be responsible for this protection. I then hypothesized that PGLYRP2 may be acting as a bactericidal molecule. Indeed, zebrafish and rockfish orthologs of mammalian PGLYRP2 have been shown to function both as amidase and bactericidal molecule against Gram-negative and Gram-positive bacteria (Kim et al., 2010; Li et al., 2007). Although the antimicrobial property of mammalian PGLYRP2 has
never been shown, the notion that mammalian PGLYRP2 also possesses antibacterial properties has recently been suggested (Royet et al., 2011). Given that neutrophils are known to have a wide range of antimicrobial arsenals and that the clearance of Salmonella was blunted in the absence of PGLYRP2 although more neutrophils infiltrated in Pglyrp2−/− mice following infection, I investigated the possibility that PGLYRP2, secreted from neutrophils, may then act as an antimicrobial molecule. It was previously reported that serum amidase, PGLYRP2, was expressed in granules of neutrophils and eosinophils (Hoijer et al., 1997a). However, by flow cytometry, I found the expression of PGLYRP2 in neutrophils in the cecal tissue to be minimal (Fig 6.2 B& C). I also performed neutrophil killing assays by isolating neutrophils from wild-type and Pglyrp2−/− mice in order to test their killing activity against Salmonella in vitro, but found that neutrophils from both wild-type and Pglyrp2−/− mice were able to kill Salmonella with similar efficiency (Figure 7.2). Another possibility might be that the antimicrobial effect of PGLYRP2 could be masked by other PGRPs such as PGLYRP1, which is constitutively expressed in the granules of neutrophils and eosinophils (Cho et al., 2005; Dziarski et al., 2003). PGLYRP1 expression was not impaired in Pglyrp2−/− mice, since I found the level of Pglyrp1 to be similar in both wild-type and Pglyrp2−/− cecum during SL1344 infection (Figure 7.3).

Future directions: In order to investigate the antimicrobial role of PGLYRP2, more direct approach where purified PGLYRP2 is incubated with Salmonella Typhimurium should be used to determine whether PGLYRP2 could inhibit the growth of the enteric pathogen. Moreover, the wild-type and Pglyrp2−/− cecal tissues following infection with Salmonella should be examined for the expression of other antimicrobial peptides such as defensins, which are known to play an important role in intestinal mucosal immunity against bacterial pathogens. Previously, the porcine amidase PGLYRP2 was suggested to also function in regulating the expression of the antimicrobial peptide β-defensin-1 (Sang et al., 2005). Thus, it would be interesting to investigate whether murine or human PGLYRP2 can regulate expression of these antimicrobial molecules.
Figure 7.2. The neutrophil killing assay.

Neutrophils were isolated from WT and Pglyrp2−/− mice and incubated with Salmonella enterica serovar Typhimurium (MOI of 100:1) at different time points (0min, 60min, 90min). Following incubation, neutrophils were lysed with 1% Triton X, and the supernatants were serially diluted and plated onto LB agar to measure CFU counts (n=3).
Figure 7.3. *Pglyrp1* expression in the cecum following *Salmonella* infection at 72h.

qRT-PCR was used to measure the expression of *Pglyrp1* in the ceca from uninfected and infected wild-type and *Pglyrp2*−/− mice at 72h. The expression is normalized to the housekeeping gene *Rpl19* (6 to 8 mice per group per experiment) (n=3). NS: not significant.
Investigating the role of PGLYRP2 in a Citrobacter rodentium-induced colitis model

In vivo studies on PGLYRP2 thus far seem to suggest diverse roles of PGLYRP2 in tissue-, and disease-specific manner. Indeed, PGLYRP2 in arthritis model was shown to play pro-inflammatory role in localized inflammatory response at the ankle joints and the feet (Saha et al., 2009). In contrast, PGRPL2 had an opposing effect in the skin, as PGLYRP2 was recently shown to have a protective role in an experimentally induced psoriasis mouse model by promoting Tregs and limiting Th17 responses (Park et al., 2011a). Interestingly, however, PGLYRP2 does not seem to have a role in inflammatory skin disease such as experimentally-induced atopic dermatitis and contact dermatitis (Park et al., 2011b). At the intestinal mucosa, PGLYRP2 seems to serve different functions depending on the model of colitis used. In a chemically-induced colitis model, PGLYRP2 has a protective role, but is not the only PGRPs with this function, as all four PGRP knockout mice (Pglyrp1−/− to Pglyrp4−/−) were shown to be more sensitive than wild-type mice to DSS-induced colitis (Saha et al., 2010). Contrary to my observations using the S. Typhimurium colitis model, my preliminary data with C. rodentium, an attaching/effacing mouse pathogen related to enteropathogenic Escherichia coli (EPEC) (Luperchio and Schauer, 2001; Schauer and Falkow, 1993), seems to offer an interesting, and yet different outcome.

Because a recent publication from our laboratory showed that Nod1/Nod2 are required to mount a robust early Th17 response to bacterial colitis in both the C. rodentium and S. Typhimurium models, I also examined the role of PGLYRP2 in the immune response against C. rodentium by analyzing Pglyrp2-deficient mice for pathological changes as well as monitoring changes in cytokine production (IFN-γ and IL-17a) by intracellular cytokine staining. Following 7-day infection with C. rodentium (1 x 10⁹ CFU) via oral gavage, Pglyrp2-deficient mice showed significantly lower pathological scores and less visible colonic inflammation compared to the wild-type mice (Figure 7.4), which followed the trend seen with the Nod1/Nod2 double KO mice (Geddes et al., 2011). My preliminary findings on the expression of intracellular cytokines suggest that baseline immune responses in Pglyrp2-deficient mice are more Th1-skewed, with higher levels of IFN-γ than WT mice (Figure 7.5a & 7.5b). Furthermore, Pglyrp2-deficient mice infected with C. rodentium at day 7 also showed enhanced IFN-γ (Figure 7.5b). In contrast, Pglyrp2-deficient mice have reduced levels of IL-17a producing CD4⁺ T cells 7 days after infection with C. rodentium (Figure 7.5a & 7.5c). All together, these preliminary data are
interesting, and seem to suggest that mammalian PGLYRP2 may trigger different immune responses against different enteric pathogens.

*Future directions:* The role of PGLYRP2 in a *C. rodentium*-induced colitis model should continue to be investigated. First and foremost, my preliminary work with *C. rodentium* should be repeated thoroughly to see whether my observations are reproducible and expandable. This will involve collecting uninfected and infected cecum/colon samples from wild-type and *Pglyrp2*−/− mice at different time points (4, 7 or 10-day) following infection with *C. rodentium* and comparing these samples for pathological scoring, bacterial loads, and cytokine production.
Figure 7.4. Colonic pathology following 7-day infection with *C. rodentium* in WT and *Pglyrp2*-deficient mice.

A) total pathological scores (top) and individual inflammation scores (bottom) are shown. Total pathological scores are composed of individual inflammation scores including submucosal edema, goblet cell depletion, epithelial hyperplasia & integrity, and inflammatory cell infiltrate.

B) hematoxylin- and eosin-stained colon samples from 7-day-infected wild-type (top panel) and *Pglyrp2*-deficient (bottom panel) mice.
Figure 7.5. Intracellular cytokines, IL-17a and IFN-γ responses from cecal WT and Pglyrp2-deficient mice during C. rodentium-induced colitis.

A) Flow cytometric analysis of intracellular cytokine release from CD4+ TCRβ+ LPLs of uninfected and 7-day infected wild-type and Pglyrp2<sup>−/−</sup> (KO) mice. Bar graphs depict the average relative frequency of all B) IFN-γ+ cells or C) IL-17a+ cells.
The effect of PGLYRP2 in maintaining normal microbiota composition in the gut

Recent studies have uncovered the important role of the intestinal microbiota in shaping the immune system of mammals. As previously mentioned, Nod2 was recently found to be an important regulator of the commensal gut microbiota in mice (Petnicki-Ocwieja et al., 2009; Rehman et al., 2011), as mutations/deficiency in Nod2 induces intestinal dysbiosis resulting in increased sensitivity to IBD. Because of its close proximity to Nod1 and Nod2 as a peptidoglycan sensing or modifying enzyme, the role of PGLYRP2 in maintaining normal microbiota composition was analyzed between wild-type and Pglyrp2-deficient littermates by qRT-PCR using group-specific 16S rRNA primers. My preliminary data reveals a difference in microbiota composition between wild-type and Pglyrp2-deficient mice on a C57/BL6 background when I performed a littermate comparison of the gut microbiota across the different genotypes. In particular, I observed statistically significant changes in microbiota species including Bacillus, Eubacterium rectale, Bacteroides, and Enterobacteriaceae (Figure 7.6). Interestingly, other studies showed that Pglyrp2-deficient mice on a Balb/c background and a C57/BL6 have altered microbiota (Duerr et al., 2010; Saha et al., 2010) although the expression of these individual bacterial species varies greatly between studies. It has been suggested that even if the mice were raised in the same animal facility, wild-type and Pglyrp2−/− mice could still display distinct microbiota if PGLYRP2 directly impacted on the bacterial flora, an effect observed in Nod2 KO mice recently (Petnicki-Ocwieja et al., 2009). For this reason, our mice were F2 littermates generated from wild-type and Pglyrp2−/− mice, and raised in specific pathogen-free environment. Thus, these other studies must be interpreted with caution since they did not use littermates and their mice were raised in a conventional facility, rather than a specific pathogen-free environment.

Future directions: More work is needed to determine the impact of PGLYRP2 in shaping the microbial communities of the gut. The next step with my preliminary data is to perform a more in depth community analysis using different tools available to identify more precise differences in microbiota composition. For example, the community fingerprinting techniques such as terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) are widely used to assess microbial community diversity and responses to changing environmental conditions (Marzorati et al., 2008; Zoetendal et al., 2008). These tools
Figure 7.6. The microbiota composition of cecum content samples from WT and *Pglyrp2*-deficient mice.

qRT-PCR of 16S rRNA for the indicated bacterial groups in cecal tissue homogenate. Tissue from three mice was analyzed in each group. The numbers relative to the total bacteria (eubacteria) present are indicated in percentages. The following bacterial groups are shown: *Eubacterium rectale* group, *Lactobacillus*, *Bacillus*, *Bacteroides*, *Segmented filamentous bacteria*, *Enterobacteriaceae*. *P<0.05; **P<0.01
would be useful, and further validate findings by the conventional qRT-PCR of group-specific 16S rRNA primers. Using bone-marrow chimeric animals, it would also be interesting to determine whether PGLYRP2 in the stromal vs hematopoietic compartment is more important in regulating the microbiota composition.

Next, it will be interesting to investigate how acute colitis in Pglyrp2-deficient mice impacts on the microbial composition of the gut. Indeed, this is extremely relevant for understanding the role of the microbiota in human IBD, especially because one of the IBD susceptibility loci, for which the susceptibility gene has not yet been identified, harbors human Pglyrp2 gene (Budarf et al., 2009). Pglyrp2 thus could be one of the human IBD susceptibility genes in this locus. In order to carry out this investigation, the microbiota changes should be examined in the wild-type and Pglyrp2-deficient mice using our well established bacteria-induced colitis models.

7.4 Conclusions

The work presented in this thesis reveals novel insights into the immune responses elicited by the peptidoglycan recognition molecules, Nod1, Nod2, and PGLYRP2, and defines these molecules as key regulators of immune responses at the level of the intestinal mucosa. Further studies will aid in delineating their implication in the pathogenesis of inflammatory diseases such as IBD, and open up new avenues for the treatment of the disease through the modulation of their functions.


provides a Salmonella enterica serovar Typhimurium colitis model that allows analysis of both pathogen and host. Infect Immun 71, 2839-2858.


best classified as X-linked familial hemophagocytic lymphohistiocytosis and not as X-linked lymphoproliferative disease. Blood 116, 1079-1082.


