Characterization of Tollip in the Interleukin-1 Receptor/Toll Like Receptors signaling pathways

Thèse de doctorat ès sciences de la vie (PhD)

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<tr>
<td>AP-1</td>
<td>Activator Protein-1</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Caspase</td>
<td>Cysteinyl aspartate-specific proteinase</td>
</tr>
<tr>
<td>CpG</td>
<td>CpG-oligodeoxynucleotides</td>
</tr>
<tr>
<td>CUE</td>
<td>Coupling of Ubiquitin conjugation to ER degradation</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase type 2</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Danger Associated Molecular Pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>DD</td>
<td>Death domain DD</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribo Nucleic Acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmatic Reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal Regulated Kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>GFR</td>
<td>Growth Factor Receptor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HA</td>
<td>Hemaglutinin</td>
</tr>
<tr>
<td>HECT</td>
<td>Homologous to E6-AP Carboxy Terminus</td>
</tr>
<tr>
<td>HEK</td>
<td>Human Embryonic Kidney</td>
</tr>
<tr>
<td>HRS</td>
<td>Hepatocyte growth factor Regulated tyrosine kinase Substrate</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of NF-κB</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL-1R</td>
<td>Interleukin-1 Receptor</td>
</tr>
<tr>
<td>IL-1RaCp</td>
<td>Interleukin-1 Receptor Associated Protein</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor of NF-κB</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun-N-terminal kinase</td>
</tr>
<tr>
<td>KD</td>
<td>Kinase Domain</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>LAMP</td>
<td>Lysosome Associated Membrane Protein</td>
</tr>
<tr>
<td>LBPA</td>
<td>lysobisphosphatidic acid</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine Rich Repeats</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation protein 88</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>Murine Embryonic Fibroblasts</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Hystocompatibility Complex</td>
</tr>
<tr>
<td>MEKK</td>
<td>MAP/ERK kinase kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse Embryonic Fibroblast</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Class</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MVE</td>
<td>MultiVesicular Endosome</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-κB essential Modulator</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappaB</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>Acronym</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PLP-2</td>
<td>phospholipase type 2</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen Recognition Receptor</td>
</tr>
<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phospho-Inositol-3-P Kinase</td>
</tr>
<tr>
<td>PI(3)P</td>
<td>Phospho-Inositol-3-Phosphate</td>
</tr>
<tr>
<td>PtdIns-P</td>
<td>phosphatidylinositol-phosphate</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribo Nucleic Acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans Golgi Network</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll /IL-1R Receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor-α</td>
</tr>
<tr>
<td>TOM1</td>
<td>Target Of Myb1</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain containing adaptor inducing Interferon beta</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF related Adaptor Molecule</td>
</tr>
<tr>
<td>TSG101</td>
<td>Tumor Susceptibility Gene 101</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen Recognition Receptor</td>
</tr>
<tr>
<td>Vps/Vsp</td>
<td>Vacuolar Sorting Protein</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicle Stomatitis Virus</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>UBA</td>
<td>Ubiquitin Associated domain</td>
</tr>
<tr>
<td>UEV</td>
<td>Ub E2 Variant</td>
</tr>
</tbody>
</table>
Characterization of Tollip in the IL-1R/TLR signaling pathways
Brian Brissoni, Department of Biochemistry-Epalinges

SUMMARY
IL-1R and TLRs are key players in innate immunity and inflammation. Tollip was identified as a component of IL-1RI, TLR2 and TLR4 signaling complexes that activate NF-κB and MAP kinase pathways. Tollip was previously shown as a negative regulator of NF-κB and MAP Kinase activation.

We have characterized the role of Tollip in IL-R/TLRs induced signaling by the analysis of the Tollip deficient mice. We showed that NF-κB and MAPK (p38, JNK, or ERK1/2) signaling appeared normal in Tollip deficient cells following stimulation with IL-1β, lipopolysaccharide (LPS), and other TLR ligands. Also IL-1β and TLRs ligands induced activation of immune cells was indistinguishable from wild-type cells. Strikingly, in Tollip deficient mice the production of the inflammatory cytokines, IL-6 or TNF-α was significantly reduced relative to control mice after treatment with physiological doses of IL-1β or LPS, whereas no difference was observed at high doses of stimulation with LPS or in LPS induced septic shock. Therefore, Tollip could be critical for regulation of optimal responses to IL-1β and LPS, in addition to its role as negative regulator of the signaling.

We also studied the role of Tollip as an endocytic adaptor for IL-1R endocytosis. We could show that IL-1R is ubiquitinatated after IL-1β stimulation, and that Tollip’s CUE domain binds IL-1RI in an ubiquitin-dependent manner. We followed IL-1R internalization and Tollip localization by confocal microscopy. Consistent with a role for Tollip in sorting of ubiquitinated IL-1RI, a significant amount of Tollip was also localized at the late endosomal compartment. We could show that Tollip is required for efficient lysosomal targeting of ubiquitinated IL-1RI. In the absence of Tollip or in Tollip deficient cells reconstituted with a Tollip mutant (defective in ubiquitin binding) IL-1RI accumulates in enlarged late endosomes. In addition, Tollip was shown to interact with, another endocytic adapter, Tom1, and both interact with IL-1RI.

In conclusion, we showed that Tollip is required for IL-1β and LPS signaling for cytokine production. In addition we showed and that Tollip has a role as an endocytic adapter, necessary for efficient trafficking and lysosomal degradation of IL-1RI.
Caractérisation de la protéine Tollip dans les voies de signalisation des récepteurs à l'IL-1 et "Toll-like".

Brian Brissoni, Departement de Biochimie-Epalinges

Résumé

Le récepteur à l' interleukine-1 (IL-1R) et les récepteurs "Toll-like" (TLRs) sont des acteurs cruciaux de la réponse immunitaire innée et de l’inflammation. La protéine Tollip a été identifiée comme étant un élément des complexes de signalisation, induits par les récepteurs IL-1RI, TLR-2 et TLR-4, qui mènent à l’activation de la voie des MAP kinases et de NF-κB. Dans de précédentes études, il a été montré que Tollip pouvait inhiber ces deux voies de signalisation.

Nous avons voulu caractériser plus précisément le rôle de Tollip dans l’activation des voies de signalisation initiées par IL-1R/TLRs en utilisant une lignée murine déficiente pour la protéine Tollip. Ainsi, en absence de Tollip, les cascades d'activation de NF-κB et MAPK (p38, JNK, or ERK1/2) ne semblent pas affectées après stimulation avec IL-1β, lipopolysaccharide (LPS) ou d’autres ligands des TLR. La réponse des cellules du système immunitaire induite par la stimulation avec IL-1β et les ligands des TLR est également comparable entre les souris sauvages et les souris défectives pour Tollip.

Par contre, dans cette lignée murine, la production de cytokines proinflammatoires IL-6 et TNFα induite par la stimulation à dose physiologique de IL-1β ou LPS, est réduite. Cependant, lors de stimulation à plus hautes doses de LPS ou pendant un choc septique induit par de LPS, cette réduction n’est pas observée. Ces résultats montrent que Tollip pourrait avoir un rôle déterminant dans l’activation optimale en réponse à l’ IL-1β et au LPS qui s'ajoute à sa fonction inhibitrice des mêmes voies de signalisation.

Nous avons aussi étudié le rôle de Tollip comme molécule adaptatrice du mécanisme endocytique d'internalisation de l’IL-1RI. Ainsi, l’ IL-1R est ubiquitiné après stimulation par l’ IL-1β, permettant à Tollip de se lier au récepteur. Cette interaction est réalisée entre le domaine CUE de Tollip et l'IL-1R via l'ubiquitine. L’internalisation et la localisation intracellulaire de l’IL-1RI et de Tollip ont été observés par microscopie confocale. En accord avec le rôle de Tollip dans le triage et la recirculation des IL-1R ubiquitiné, une quantité importante de Tollip été détectée dans l’endosome tardif. Nous avons pu démontrer que Tollip était nécessaire pour dirigir efficacement l’IL-1RI ubiquitiné vers les lysosomes. Dans des cellules déficientes pour Tollip, ou reconstituées avec un mutant de Tollip (MF/AA) incapable de lier l’ubiquitine, l’ IL-1RI s'accumule dans des vesicules anormales de l'endosome tardif.

Dans ce travail, nous avons pu confirmer et préciser la fonction de la protéine Tollip dans l’activation de la production de cytokines induites par l’ IL-1β and le LPS lors de l’inflammation et découvrir son rôle d'adaptateur dans l’internalisation et l'endocytose de l’ IL-1RI.
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General Introduction
1. Immunity and inflammation

1.1 The immune response

All evolved organisms are constantly exposed to a wide variety of infectious microorganisms, such as bacteria, viruses and parasites, as well to chemical or physical insults attempting to damage the integrity of the whole organism or tissues. To face pathogens and stress, the organism has developed a system of defense, the immune system. The immune response is composed of the innate and the adaptive immunity, both systems showing specific properties and characteristics (Table 1).

The innate immune response is derived from the ancient immune system of invertebrates, such as worms and insects. In mammals, as well invertebrates, the innate response is activated by germ-line fixed receptors recognizing pathogen molecules and leads to the rapid activation of anti-microbial responses. The innate response occurs within the first hours of infection to rapidly clear tissues from invading microorganisms, and activate an inflammatory response \(^1,2\).

400 million years ago vertebrates developed a sophisticated and specific immune response, the adaptive immunity. Specialized cells, the lymphocytes, mediate this response. These cells are selected for specific pathogen polypeptide (antigens) recognition. The activation of the lymphocytes induces appropriate cellular and humoral responses to eliminate invading pathogens and prevent future infections of the same microorganisms establishing memory cells.
Table 1: Difference between innate and adaptive immunity (adapted from Janeway et al.; 2002).

<table>
<thead>
<tr>
<th>Property</th>
<th>Innate immune system</th>
<th>Adaptive immune system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptors</td>
<td>Fixed in the genome</td>
<td>Genes encoded in segment for somatic recombination</td>
</tr>
<tr>
<td>Cells</td>
<td>Innate cells (macrophages, neutrophils,…)</td>
<td>Lymphocytes</td>
</tr>
<tr>
<td>Distribution</td>
<td>Non-clonal</td>
<td>Clonal</td>
</tr>
<tr>
<td>Recognition</td>
<td>Conserved molecular patterns</td>
<td>Polypeptides and proteins</td>
</tr>
<tr>
<td>Action time</td>
<td>Immediate activation (1-96h)</td>
<td>Delayed activation (from 96h)</td>
</tr>
<tr>
<td>Response</td>
<td>Antimicrobial molecules and enzymes Proinflammatory Cytokines (IL-6, IL-1β, TNFα) Chemokines (IL-8)</td>
<td>Cell proliferation and activation Effector cytokines (IFNγ)</td>
</tr>
</tbody>
</table>

Figure 1. Schematic representation of the immune response, composed of innate and adaptive immunity. (modified from Immunobiology, Janeway)
1.1.1 The Innate immunity

The first line of defense against pathogens are the epithelial and mucosal tissues, which act as a physical barrier. When the epithelial/mucosal barrier is compromised by pathogens, the organism can mount a rapid and efficient innate response to fight the invading microorganism.

The innate response is triggered by recognition of pathogens mediated by the epithelial and endothelial cells together with resident innate immune cells, including macrophages, neutrophils, phagocytic leukocytes, APCs (Antigen Presenting Cells) (i.e. macrophages, dendritic cells). These cells sense a pathogen via Pathogens Recognition Receptors (PRRs). PRRs signaling in these cells triggers ultimately the release of soluble factors (i.e. cytokines, chemokines) that orchestrate the recruitment and the activation of innate cells, to eradicate the pathogens before they spread in the organism.

1.1.1.1 Pathogens/danger recognition

The innate immunity is based on the discrimination of microorganisms or potential dangerous agents from the rest of the organism. The “self-non self” theory, proposed by Janeway and collaborators during the last two decades, is based on recognition of conserved microbial structures, also called Pathogen-Associated Molecular Patterns (PAMPs)\(^1\). The PAMPs are microbial molecules absent in the eukaryotic organisms that are essential for the microorganism, such as structural molecules of the bacterial wall (lipopolysaccharide, peptidoglican and lipoproteins), unmethylated DNA, viral DNA and RNA. PAMPs are recognized by a limited number of PRRs expressed by innate immune cells\(^3\). The PRRs are expressed as extracellular, intracellular or secreted receptors. The “self-non self” model proposes that PRRs genes evolved with the pathogen genes expressing the specific PAMPs.

Recently the “danger model” was proposed to enlarge the concept of innate immunity. This model proposes that innate immunity is induced not only by molecular patterns of pathogens but also host-derived molecular patterns from altered “self” molecules, called Danger Associated Molecular Pattern (DAMPs)\(^4\). DAMPs are molecular patterns that are not present in healthy tissues, but exposed in stressed and damaged tissues, or during infection. This model is supported by the discovery of a large panel of host derived molecules released by damaged or stressed cells and recognized by several PRRs as DAMPs.\(^5\) The PRRs are a heterogeneous group of receptors and proteins\(^1,6\), including the well studied Toll like Receptors (TLR), TLR4 co-receptor (e.g. CD14)\(^7\), leukocyte integrins (CR3, CD11/CD18), glycans and mannose receptors, and scavenger receptors (e.g MARCO, SRA)\(^8,9\). Recently,
other intracellular PRRs were identified such as NALPs 10 and NODs 11. The PRRs are expressed by monocytic cells (macrophages, neutrophils, eosinophiles) and dendritic Cells (DC), and also to some extent by many other cell types including endothelial and mucosal epithelial cells.

1.1.1.2 Innate immunity induced response

The recognition of PAMPs by the PRR (i.e. TLRs) induces several signaling pathways (i.e. NF-κB, MAPK), leading to induction of numerous genes necessary for the immune response (i.e. costimulatory molecules, MHC) and inflammation (see section 1.2 Inflammation). The rapid expression and release of general proinflammatory “alarm cytokines” (TNFα, IL-6, IL-1β) is essential to indirectly propagate the proinflammatory signal from the site of infection to the tissue. The proinflammatory response is characterized by the massive recruitment by extravasation of innate effectors cells (phagocytes). The phagocytes (i.e. neutrophils, macrophages) are specialized cells able to eliminate invading microorganisms by phagocytosis. Phagocytosis is initiated by recognition and binding to the pathogen, followed by engulfment and digestion in the lysosome 9,12. In addition active phagocytes are responsible for release of a variety of molecules in response to infection including reactive species (ROS, NOS), inflammatory lipid mediators and antimicrobial molecules 13 (Fig. 2). The innate response via phagocytosis of pathogens and the PRR stimulation also prime the adaptive response activating the APC pathogens uptake and costimulation 14-16(Fig. 2).
Figure 2. Schematic representation of the innate immunity
(1) Pathogens crossing the epithelial/mucosal barrier (1a) or tissue stress (1b) are recognized by APC (3). (2) this activates phagocytosis and anti-microbial molecules production. (4) Activation of APC leads to pathogen uptake and processing inducing APC maturation. (5) Mature APC are able to uptake, process and present pathogens molecules to naïve adaptive immunity cells, activated with adequate co-stimulatory molecules. (6) Active APC and phagocytes release proinflammatory molecules inducing a recruitment of innate immunity cells to damaged tissue (7) and general proinflammatory response (8).
1.1.2 The adaptive response

The innate immune response in human is immediate (from 4h to 96h) to pathogens using preformed and transiently synthesized effectors molecules, and phagocytes. However, the innate response can be easily overwhelmed by infecting microorganisms: in this case the organism mounts delayed antigen-specific humoral and cellular responses, namely the adaptative immune response.

The adaptative response is based on recognition of pathogen antigens presented by the Antigen Presenting Cells (APC). The antigens are the polypeptides processed by proteolytic activity of APC and that can induce antibody production (Immunobiology, Janeway).

The antigen-specific receptors expressed by the lymphocytes include immunoglobulins (Ig), Major Histocompatibility Complex (MHC), B and T Cells Receptor (BCR, TCR) expressed by lymphocytes. The genes of these antigen-specific receptors undergo somatic recombinations in the lymphocytes forming a large population of naive lymphocytic clones, each carrying different antigen-specific receptors.

During the infection, Antigen Presenting Cells (APC) (i.e. DC, macrophages) take up and present antigen peptides on their surface on the MHC molecules. In the same time activation of the PRR (i.e. TLRs) by the pathogen on APCs induces the expression of surface costimulatory molecules (ex. B7.1, B7.2/CD86) (Fig.2 and 3)\textsuperscript{16}.

Naive T (maturated in the thymus) and B lymphocytes (maturated in bone marrow) receiving antigenic signal (antigens-MHC) together with the costimulatory signal from APCs are selected for proliferation, whereas the non-selected cells undergo anergy and apoptosis\textsuperscript{1,17,18}.

These selected antigen-specific lymphocytes proliferate, mature and become active effectors of an immediate and efficient response against antigens, and form memory cells. The antigen-specific T cells can mature to cytotoxic T lymphocytes (CD8\textsuperscript{+}) or helper T lymphocytes (CD4\textsuperscript{+}), giving rise to a cellular response. The antigen-specific B cells are clonally selected for antigen-specific antibody production and mature to plasma cells and memory cells, leading to an humoral response (Fig. 3).
Figure 3. Schematic representation of the adaptive immunity.

1. Activation of APC and antigen uptake induces inflammation (2) and the migration of mature APC to lymphoid organs.
2. Mature APC present the pathogens antigen loaded on MHC recognized by naïve T and B cells.
3. Antigen specific B lymphocytic clones proliferate and differentiate into plasma cells, able to produce specific antibodies against the pathogen to mount an humoral response.
4. Antigen specific T lymphocytic clones proliferate and differentiate into subset of specific cells: the cytotoxic effectors T cells (CD8⁺) responsible for the cellular response, and the helper T cells (CD4⁺) contributing to humoral response.
1.2 The Inflammatory response

The inflammatory process is vital for the survival of all evolved organisms, and plays a crucial role in health and diseases. Inflammation is defined as localized or general defense in response to presence of pathogens, chemicals or physical damages, leading to elimination of the injurious agent and healing of the affected tissues. The inflammatory response orchestrates the activation of both, innate and adaptative immunity.

Inflammation is characterized by the classic signs such as pain (dolor), heat (calor), redness (rubor), swelling (tumor) and loss of function. These macroscopic effects of inflammation involve a complex series of events mediated by cellular responses and chemical signals.

The initiation of inflammation is induced in injured or stressed tissues by antigen-specific recognition (antibody, TCR) and/or by non-specific recognition by phagocytes or the complement pathways. Inflammation induces blood vessel dilatation, increased blood flow and permeability, exudation of fluids and extravasations of inflammatory cells (neutrophiles, macrophages, mast cells) into the inflammatory focus. These tissue and cellular effects go together with the release of proinflammatory molecules.

The activation of inflammation at the molecular level is mediated by the PRRs (i.e. TLRs) and cytokine receptors (e.g interferon and interleukin receptors) leading to activation of a complex web of transcription factors. These transcription factors (NF-κB, AP-1, and IRFs) induce the expression of a large panel of genes, playing a role in tissular and cellular inflammatory reactions including proinflammatory cytokines (TNFα, IL-6, IL-1β, IL-18, IL-12p40, INF-β, GM-SCF), adhesion molecules (V-CAM, I-CAM, integrins, selectin) and attractants chemokines (IL-8, MCP-1, RANTES), proinflammatory enzymes (i.e. COX-2, PLP-2), inflammatory lipids (prostaglandins and leukotrienes), production molecular reactive species (NO, ROS), antimicrobial molecules(i.e. defensin2) and tissue degrading enzymes (i.e. MMP)^13.

Inflammation orchestrates the immune response, tissue healing as well as allergic reactions and inflammatory diseases. For this reason, inflammation is a fine tuned process, regulated in magnitude, space and in a limited period by balancing between the proinflammatory and anti-inflammatory signals. An insufficient inflammatory response results in immunodeficiency, infection and cancer. In contrary excessive inflammatory response causes fever and diffuse pain, as well as autoinflammatory and autoimmune diseases^20.
2. The TIR Receptors superfamily

2.0. Introduction

In the 1990s the first TIR Superfamily member was identified, the *Drosophila* Toll receptor, characterized by the presence of a specific motif, the Toll/IL-1R (TIR) domain. Subsequently others TIR containing proteins were identified in the mammalian genome.

The TIR domain is a 200 amino acids motif conserved in plants, insects and mammals during evolution \(^21\) (Fig. 4). The TIR domain structure contains three conserved boxes. Box1 is the signature sequence of the TIR domain family; Box2 and Box3 contain crucial residues for signaling. The conserved sequence and structure similarity of the TIR domain to bacterial chemotaxis regulator (CheY) and the presence of TIR-like proteins in *Streptomyces coelicor* \(^21\) suggest that TIR structure evolved from unicellular ancestors. TIR domain proteins are conserved all along the evolution from plants (ex. RPP1, RPP5) \(^22,23\), through insects (i.e. dToll) \(^24\), to mammals.

The proteins of the TIR Superfamily are subdivided in three subgroups: receptors with extracellular LRR, receptors with extracellular Ig-like domains and intracellular TIR-containing proteins (Fig. 4).

Interestingly, the TIR receptor superfamily members are key players of innate immunity and proinflammatory signals. The activation of TLRs by PAMPs or IL-1RI by IL-1\(\beta\) activates common downstream signaling cascades (i.e. NF-\(\kappa\)B, MAPK, IRFs) \(^25\).
Figure 4. Distribution of TIR containing proteins in animals and plants.
2.1 The Toll Receptors

The first discovered TIR containing receptor, the *Drosophila* Toll receptor was originally described as a developmental factor in the dorsal/ventral polarity in the larvae. Later its crucial role in host defense against fungal infection in the adult fly was discovered. The Toll receptor has an extracellular part with a Leucine Rich Repeat (LRR), composed of different numbers of a repeated xxLxLxx motif and other conserved leucines. Intracellularly, the Toll receptor has a TIR domain. Evolutionary and structural studies show that LRR has high variability and ability to bind different motifs or molecules. In insects Toll Receptor signaling mediates the defense against pathogens, based on production of antimicrobial peptides and activation of a systemic response. The cognate ligand of the Toll receptor is Spätzle which is produced as an inactive proform. Serine proteases in the haemolymph, activated by the presence of pathogenic microorganisms, cleave Pro-Spätzle into the active form, Spätzle. The binding of Spätzle on the Toll receptor results in the initiation of the Tube/pelle/Dtraf/Cactus signaling pathway and activation of the Dif/Relish transcription factors. Activation of Dif/Relish leads to induction of genes encoding antimicrobial compounds, such as drosomycin responsible for anti-fungal response. The mammalian orthologues of these signaling cascade are MyD88/IRAK-1/TRAF6/IκB and p65/p50 NF-κB transcription factors, respectively. There are three further *Drosophila* TIR-receptors members, 18-Wheeler that plays a role in antimicrobials response, and Mst-Prox and Tehao that have an undefined function.

2.2 Mammalian Toll-like Receptors and their ligands

In 1997, a mammals gene similar to the *Toll* gene was identified through a bioinformatic approach and was called Toll-like receptor (TLR). It was initially demonstrated that this gene product promotes the expression of genes encoding inflammatory cytokines, suggesting that TLR in mammals also has a function in innate immune responses as PRR. Later other genes homologous to the *Toll* gene were identified, and these genes were referred to as the Toll-like receptor (TLR) family. So far, 12 members in mice and 10 in human of the TLR family have been identified.

The first evidence of TLRs in the recognition of pathogens was reported from studies on mice with disrupted *Tlr4* gene. These mice are unresponsive to bacterial lipopolysaccharide (LPS), an integral component of the outer membrane of Gram-negative bacteria that can cause endotoxin shock.
Subsequently, biochemical studies demonstrated that TLR4 requires as co-receptors, the (GPI)-anchored molecule CD14\textsuperscript{36} and MD-2 protein that interacts with the extracellular portion of TLR4\textsuperscript{37,38} for optimal signaling.

The generation of TLRs knockout mice for each TLR gene has revealed which pathogen(s) can be recognized by which TLR. TLR2 is involved in responses to LTA (LipoTechoic Acid), lipoproteins/lipopeptides, glycosyl-phosphatidylinositol anchors from \textit{Trypanosoma cruzi}, and zymosan\textsuperscript{15,39}. However, recognition of these TLR2 ligands requires another TLR family member as coreceptors. The mycoplasmal diacylated lipopeptide MALP-2 is recognized by a heterodimer of TLR2 and TLR6, whereas the bacterial triacylated lipopeptide PAM3CSK4 is recognized by a heterodimer of TLR2 and TLR1\textsuperscript{40,41}. TLR5 recognizes flagellin, the main protein monomer constituting bacterial flagellum, the polymeric rod-like appendage extending from the outer membrane of Gram-negative bacteria, is also a potent pro-inflammatory inducer\textsuperscript{42}. TLR3 recognizes double-stranded (ds) RNA that is generated in the lifecycle of RNA viruses during infection\textsuperscript{43,44}. TLR7 and TLR8 recognizes single stranded DNA (ssDNA) and pharmaceutical compounds imiquimod and resiquimod\textsuperscript{45,46}. These compounds of the imidazoquinoline family are known to have potent antiviral and antitumor activities. TLR7 and its close relative TLR8 also recognize single-stranded RNA present in numerous viruses\textsuperscript{45,46}. TLR9 recognizes unmethylated 2'-deoxyribo(cytidine-phosphate-guanosine) (CpG) DNA motifs commonly present in bacterial and viral genomes that have immunostimulatory activities\textsuperscript{47,48}. It has recently been shown that TLR11 in mouse, which is abundantly expressed in the kidney and bladder, senses uropathogenic bacteria\textsuperscript{49}.

However, recently it was shown that the TLRs also have the role as receptors recognizing endogenous molecules released during stress conditions (danger signals). The pathogens and endogenous ligands of TLRs are summarized in Table 2.
<table>
<thead>
<tr>
<th>Toll-Receptor</th>
<th>Co-receptor</th>
<th>Exogenous ligands</th>
<th>Endogenous Ligands</th>
<th>Reference</th>
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<tbody>
<tr>
<td>TLR1</td>
<td>TLR2</td>
<td>Lipopetides</td>
<td>Hsp60, Hsp70</td>
<td>Hajjar et al. (2001)</td>
</tr>
<tr>
<td>TLR2</td>
<td>TLR1, TLR6, CD11/18</td>
<td>PGN, lipoproteins, lipotoxin acid, Zimosan, glycolipids dsRNA, poly(I:C)</td>
<td>Schwander et al. (1999)</td>
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<tr>
<td>TLR3</td>
<td></td>
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<td>Aliprantis et al. (1999)</td>
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<tr>
<td>TLR4</td>
<td>CD14, MD-2</td>
<td>LPS, LTA, Taxol</td>
<td>Hsp60, Hsp70, Fibronectin, Hyaluronic acid</td>
<td>Axenopoulou et al. (2001)</td>
</tr>
<tr>
<td>TLR5</td>
<td></td>
<td>Flagellin</td>
<td></td>
<td>Poltorak et al. (2001)</td>
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<td>TLR7</td>
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<td>Gewirtz et al. (2001)</td>
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<td>Bulut et al. (2001), Hajjar et al. (2001)</td>
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<td>Cpg</td>
<td>DNA</td>
<td>Jurek et al. (2001)</td>
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<td>TLR10</td>
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<td>Hemmi et al. (2001), Takeshita et al. (2001)</td>
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<tr>
<td>TLR11</td>
<td></td>
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<td>Zhang et al. (2004)</td>
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Table 2. The mammals Toll-like receptor and selected pathogen-derived (exogenous) and host-derived ligands (endogenous).
2.3 The IL-1R-like Family and ligands

The interleukin (IL) family consists of cytokines with a characteristic three foiled-shape structure and is composed of several members: IL-1β, IL1α, IL-1 Receptor Antagonist (IL-1ra), IL-18, and six newly discovered members named IL-1F5 to IL-1F10. The remarkable conservation between diverse species from birds to mammals indicates that the IL-1 system represents an ancient signaling pathway critical for responses to environmental stresses and attack by pathogens. Knockout mice and biochemical studies show the pivotal role of interleukin-1 related cytokines in mammalian immune responses and inflammation.

The interleukin-1 related cytokines bind receptors of the IL-1R-like family, a large family of TIR containing type I transmembrane receptors. The prototypical member of IL-1R like family is the IL-1R Type I (CD121). Closely related to the IL-1RII (a decoy receptor for IL-1RI) and the its co-receptor IL-1RAcP. The IL-1R-like family also includes the IL-18R and co-receptor IL-18RAcP, responsible for IL-18 signaling, the orphan receptors T1/ST2, IL-1Rrp2, IL-1RAPL and SIGGIR. IL-18 was described as an IFN-γ inducing gene, crucial activator of TH1 cells and natural Killer cells (NK). The IL-18R has two related proteins, its co-receptor IL-18RAcP and the orphan receptor IL-1Rrp2. The gene of the receptor named T1/ST2 was cloned as a delayed response gene to proliferative signals and is present as a membrane bound or soluble form. There are also soluble and membrane bound forms of T1/ST2. The Single Ig Receptor (SIGGIR) is an inhibitor of IL-1R induced signaling in epithelial tissues and lymphoid cells. The receptor IL-1RAPL is similar to IL-1RAcP and was found as a gene affected in X-linked mental retardation, and linked with cognitive and mnemonic defects. All this section reviewed in.

2.3.1 Interleukin-1

Interleukin-1 (IL-1) is a pro-inflammatory “multifunctional” cytokine produced by activated macrophages and monocytes. It is interesting to note that IL-1 is found not only in mammals but also in birds, amphibians, bony fish and cartilaginous fish. The conservation of IL-1 throughout evolution says much about the importance of this cytokine for evolved organisms. IL-1 has two isoforms, IL-1α and IL-1β. It was shown that IL-1α and IL-1β do not have identical biological effects, although they share a common receptor. Where IL-1α is a potent activator of the humoral immune response, IL-1β is the predominant form for inflammatory responses. The mRNA of IL-1 is present in almost all tissues, but is translated into the immature cytosolic forms pro-IL-1β and proIL-1α (31 kDa) in a stimuli and cell type specific
manner. The Pro-IL-1β is cleaved by Caspase-1/ IL-1β converting enzyme (ICE) to give a 17 kDa mature form and secreted by an unknown mechanism into the extracellular environment 57. IL-1β is mainly produced by monocytes (macrophages, Neutrophiles), whereas IL-1α is produced principally by keratinocytes 58-60. Originally, IL-1β was described as “lymphocytes-activating factor” due to its property to induce maturation and proliferation of lymphocytes 61. Further clinical studies showed the role of IL-1β in inducing physiological effects such as hypotension, fever and sleepiness, general inflammation and pain 62.

Today it is clear that IL-1β is a potent proinflammatory mediator and regulator of the immune system. IL-1β is released in response to microbial infections (viral, bacterial, fungal, and parasitic) and in non-infectious diseases (tumors, immunodeficiency, autoimmune disorders, pathogenic trauma), physical insults damage (hypoxia, tissue damaging, host graft), or upon stimulation with others cytokines (TNFα, IFN-γ, GM-CSF and IL-2), as well as T-cell/antigen presenting cell interactions and immune complexes (antibodies).

The binding of IL-1β to cognate surface receptor (IL-1RI) activates a complex web of transcription factor, such as NF-κB, AP-1, and IRFs, together with many others signaling cascades. These signaling pathways induce the expression of a large panel of genes involved in inflammation and immunity, including IL-1β, IL-6, TNFα, IL-12p40.

Due to the crucial role of IL-1β in inflammation deregulation of its activity leads to chronic inflammatory diseases such as autoimmune diseases (i.e. rheumatoid arthritis, asthma), and in periodic fever disease 63,64.

2.3.2 The Interleukin-1 Receptors

The IL-1R-like are characterized by the presence of an intracellular TIR domain and an extracellular region composed by three Ig-like domains. Both IL-1β and IL-1α are recognized by two different chains: IL-1RI and IL-1RII. Only IL-1RI contains an intracellular TIR domain responsible, with the co-receptor IL-1RacP, for IL-1 induced signal transduction 65. In contrast the IL-1RII has a truncated intracellular domain with no signaling capacity and appears to act as decoy receptor 66,67. In IL-1RI-deficient mice, IL-1 responses such as increased IL-6 production and fever are impaired. Also impaired was the acute-phase response, delayed-type hypersensitivity, and the ability to combat infection by Listeria. These results all indicate the importance of IL-1RI for inflammation and infection 68. It was also observed that non-transformed or primary cells express few IL-1RII copies (100-200 copies per cells) which is sufficient for efficient IL-1 mediated signaling 69.
The resolution of the crystal structure of the extracellular Ig domain with IL-1 showed that the two first Ig domains bind tightly to IL-1, while the third is wrapped around the ligand, allowing contact with the co-receptor, IL-1RAcP. The co-receptor IL-1RAcP does not bind IL-1, but increases the affinity of IL-1RI for IL-1 and is necessary to trigger IL-1 induced signaling \cite{70,71}.
2.4 TIR Receptor superfamily signaling

2.4.1 MyD88-dependent and independent signaling

The receptors of the TIR superfamily signal through conserved pathways. Ligand binding to TLR or IL-1R-like induces the formation of a heterodimeric (consisting of the receptor and its co-receptor) or homodimeric receptor complex. The TIR domain present in all these receptors is responsible for initiating multiple signaling cascades through homophilic (TIR-TIR) interactions between TIR containing proteins. As a consequence, the TIR domains serve as scaffolds platforms, recruiting TIR-adaptors (MyD88, TIRAP/mal, TRIF and TRAM). Recruitment of one or multiple TIR-adaptors allows the formation a signaling module triggering multiple pathways (i.e. NF-κB, AP-1, IRFs).

The best-characterized TIR-adapter is MyD88 (Myeloid Differentiation 88), containing a TIR and a Death domain (DD). The MyD88-dependent signaling pathway activates the signalosome of IRAK-1/IRAK-4/TRAF6 leading to NF-κB and AP-1 activation, crucial for the expression of pro-inflammatory genes. MyD88 is essential for all IL-1R-like receptors and for the majority of the TLRs, as shown by the analysis of MyD88-deficient mice. MyD88-deficient mice are completely defective in the production of inflammatory cytokines such as TNFα, IL-6, IL-1β, and IL-12 p40 in response to IL-1β and certain TLRs (TLR5, TLR9). In addition, MyD88-deficient cells are not able to proliferate in response to stimulation with LPS, CpG DNA, or IL-1β.

However, MyD88-deficiency does not abolish but delays TLR4 and TLR2 mediated NF-κB and MAPK activation, where the IRF pathways remain intact. Indeed these pathways are mediated by other TIR-adaptors, (i.e. TIRAP/Mal TRIF and TRAM), that induce typeI interferon (IFN)-inducible genes, such as IP-10, GARG-16, or IRG-1, and surface markers, including CD40, CD80, or CD86.

These observations indicate that TIR receptor signaling cascades, can be separated into two pathways: a MyD88-dependent pathway that leads to the production of pro-inflammatory cytokines with quick activation of NF-κB and MAPK, and a MyD88-independent pathway associated with the induction of IFN-inducible genes in certain TLRs.
2.4.2 IL-1R/TLR Signaling pathways

The recruitment of MyD88 to the TIR domains of the receptor complex induces the rapid translocation of a serine/threonine kinase containing a DD, the IL-1R Associated Kinase 1 (IRAK-1) to the IL-1RI 82,83. This brings the death domains of IRAK-1 and MyD88 into close contact. It was also shown that interaction of IRAK-1 with IL-1R mediated by Tollip, that is in turn recruited to IL-1RacP upon IL-1β stimulation 84. The recruitment of IRAK-1 to the receptor complex, rapidly leads to phosphorylation induced by another kinase that is also binds via MyD88, IRAK-4. IRAK-4 was shown as the kinase that initially phosphorylates IRAK-1 in the activation loop, leading to autophosphorylation of IRAK-1 85. In contrast, to the IRAK-1 deficiency, that shows only a partial defect in NF-κB activation 86,87, IRAK-4 deficiency completely blocks signaling by IL-1R, IL-18R and all TLRs 73. Another member of the IRAK family recruited to IRAK-1, IRAK-M is an inducible negative regulator of the TLR/IL-1R signaling in macrophages 88.

IRAK-1 recruits TRAF6 that is also associated to a complex consisting of TAK1, TAB1 and TAB2 72,89,90. The formation of this multiprotein signalosome formed by TRAF6/TAK-1/TABs, presumably induces the formation of K63 ubiquitin polychains, due to the recruitment of an enzyme complex (E2) consisting of Ubc13 and Uev1A, and the presence of the TRAF6 RING domain that functions as an ubiquitin ligase (E3) 91,92. The fate of IRAK-1 is to undergo ubiquitination and targeting towards proteosomal degradation, suggesting that IRAK-1 degradation allows dissociation of the signalosome complex around TRAF6, limiting the duration of the signal 93.

Activated TRAF6 signalosome activate the MAPKKK, TAK1 94. TAK1 activate two divergent cascades through MAP Kinase and IKKs, that lead to transcription factors AP-1 and NF-κB respectively 72.

The formation and activation of the IKK complex is induced by the activated platform TRAF6/TAK1. The IKK complex is composed of the regulatory subunit NEMO and two active subunits IKK-α and IKK-β, that are activated by phoshorylation. IκBα keeps the NF-κB subunits inactive in the cytoplasm. The active IKK complex phosphorylates the inhibitor of NF-κB (IκBα), leading to its ubiquitination and proteosomal degradation. The IκBα degradation allows the p65/p50 NF-κB subunits to translocate into the nucleus and engage transcription (Fig.5).

TAK1 can activate down stream MEKK3 and MEKK6 to phosphorylate p38, but also MEKK4 and MEKK7 to phosphorylate JNK, and indirectly MEKK1 for Erk-1/Erk-2 phosphorylation.
The phosphorylation cascades induce the activation of several transcription factors, principally jun-Fos/AP-1, ATF2, TCF, MEF2C and Elk-1 (reviewed in Kyriakis et. al 2001) 95. Another adaptor, Ecsit, appears to be involved in MEKK1 activation, leading to phosphorylation of JNK, p38 MAPKs, but also IKK 96. Also crosstalk between MAPK and IKK activation was shown in TLR and IL-1R signaling mediated by MEKK3, as shown by MEKK3 Knockout mice analysis 97 (Fig. 5).

The MyD88-independent pathways are activated using others TIR-adaptors such as TIRAP/MAL 98,99 and TRIF/TICAM1 and TRAM 100-103. These TIR adaptors lead to formations of alternative signaling complexes that initiate MyD88-independent rapid activation of NF-κB (e.g TIRAP/Mal), delayed NF-κB activation (TRIF), and activation of Interferon Responding Factor (IRF) in TLR2, TLR3 and TLR4 signaling 72,104. The IRF signaling cascades is mediated by TRIF and TRAM adaptors, which activate IRFs. The IRF3 is activated via the non-canonical IKK (IKKe) and TBK1 kinase to induce INFα/INFβ and also proinflammatory genes. Induction of the IRF3 transcription factor leads to type I interferon INFβ production and paracrine activation of the JAK/STAT pathway via interferon receptors 72,81,104,105. IRF7 was shown also shown be important for MyD88 dependent and independent production of INFα/INFβ infection and by TLR7 and 9 stimulation 106-108. However, IRF5 knockowut mice analysis and others studies showed that IRF5 via MyD88 and TRAF6 induce proinflammatory cytokine but is not essential for INFα production 107,109.

Recently, it was shown that LPS and IL-1β activate other pathways involved in the regulation of several cell processes including the activation of phosphatidylinositol-3 kinase (PI-3-K) via IRAK-1 and Rac and subsequently of protein Kinase B (Akt) 110, activation of acidic and neutral sphingomyelinases to produce ceramides, GTP-binding proteins, protein kinase C forms phospholipases (PKC), and phospholipases activation. All this is reviewed in Martin, et al., 2002 111.
Figure 5. Representation of the principal TIR superfamily Receptors (TLRs, IL-1R/IL-18R) signaling pathways and transcription factor activation.
Chapter 1:
Tollip deficient mice characterization
1. Introduction: Tollip (Toll Interacting Protein)

1.1 Tollip structure and features

The Tollip protein (Toll-interacting protein) was first identified by means of a yeast two-hybrid screen using a murine cDNA library and the cytoplasmic tail of IL-1RAcP (residues 385–570) as bait. The murine cDNA Tollip (850bp) encodes a protein of 274 amino acids with an apparent size of 28 kD that is ubiquitously expressed. Database searches identified counterparts of Tollip in humans and in Caenorhabditis elegans, sharing 97% and 41% sequence identity, respectively. (Fig.6). All Tollip orthologs seem to have conserved domain architecture, with a C2 domain and a C-terminal CUE domain (Fig. 1.1).

The residues from amino acid 54 to 186 of Tollip correspond to a known sequence motif, namely the C2 domain. The C2 Domain is a motif of approximately 130 residues in length identified in a growing number of eukaryotic signaling proteins C2 domain interact with cellular membranes and mediate a broad array of critical intracellular processes, including membrane trafficking, the generation of lipid-second messengers, activation of GTPases, and the control of protein phosphorylation. The C2 domains also display the remarkable property of binding a variety of different ligands and substrates, including Ca$^{2+}$, phospholipids, inositol polyphosphates, and intracellular proteins. Sequence alignment of C2 domains predicts that they form two distinct topological folds related to synaptotagmin 1 and phosphoinositide-specific phospholipase C-δ1, respectively. The C2 domain of Tollip seems to be of type-II topology (similar to C2 domain of phosphoinositide-specific phospholipase C-δ1). Like some others members of the type II class, Tollip’s C2 domain is not predicted to be regulated in a Ca$^{2+}$-dependent manner, as two of the five aspartate residues normally required for Ca$^{2+}$ binding are absent in Tollip. Recently it was shown that the C2 Domain of Tollip binds phosphatidylinositol-3-phosphate (PtdIns(3)P) and phosphatidylinositol-3,4,5-phosphate (PtdIns(3,4,5)P) in vitro. The C-terminal region of Tollip contains a motif of 54 amino acids identified as a CUE domain, a novel ubiquitin-binding domain. The features and function of the CUE domain will be described in a separate section (see 1.3, chapter II).

1.2 Tollip and TLR/IL-1R signaling

Tollip was shown to interact with IL-1RI complex upon overexpression and to be rapidly recruited to the activated IL-1RI complex after IL-1β stimulation. Later, recruitment of Tollip to TLR2 and TLR4 after stimulation with cognate ligands was also shown. These
studies revealed a potential role of Tollip as a negative regulator in TLRs and IL-1RI signaling. In fact, Tollip overexpression dramatically suppresses NF-κB activation in luciferase assays \(^{84,113,114}\), and it inhibits the kinase activity of IRAK-1 \(^{113}\). Tollip was found to form a complex with IRAK-1 in resting cells and to be recruited with similar kinetics to the IL-1R complex \(^{84}\). This led to the idea that Tollip may be involved in IRAK-1 recruitment and in the maintenance of IRAK-1 in an off-state prior to receptor triggering \(^{84}\). The C2 domain of Tollip that bind to PtdIns(3)P (PI3P) was also found to be involved in Tollip inhibition of the IL-1 and LPS signaling \(^{115}\).

In addition, others studies show a specific upregulation of Tollip mRNA level and protein expression in intestinal epithelial cells (IEC) and THP1 cells following LPS stimulation, suggesting that Tollip could have a suppressive function on signaling in LPS stimulated tolerant cells due to continuous TLR signaling \(^{115,116}\).

**Figure 1.1 Tollip domains and sequence**

a. Tollip domain architecture, containing C2 and CUE domains. b. The alignment of Tollip orthologs in mammals and worms, showing conserved features in the secondary sequence.
2. Material and methods

Tollip mice generation and screening
Genomic DNA containing the Tollip gene was isolated from mice genomic BAC library. The targeting vector was constructed by replacing the region of 11.5 (kb) genomic fragment encoding the amino-terminal region of Tollip containing the first exon start codon (1kb) with a neomycin resistance cassette (neo). The targeting vector was given to Anteq (Switzerland) for the generation of Tollip deficient mice. The Tollip targeting vector was transfected in embryonic stem cells for homologous recombination. Homologous recombinant Neomycin resistant cells were verified by Southern Blot, then microinjected into blastocytes of SV129 mice. Chimeric mice were mated to obtain heterozygous F1 progeny which were then intercrossed to obtain Tollip+/- mice. The Tollip+/- homzygous progeny were backcrossed four times into C57BL/6 background. Tollip+/- mice were screened by Western Blot with rabbit anti-Tollip antibody or by PCR using on genomic DNA and the following primers for Tollip gene 5' GGATTTGGGATTTCATCAGAGGC3' (JT2886), 5'ACAAGAGTGGGACGGAACCTTC3' (JT2887), and JT2886 with GGAGAGGCTATTCGGCTATG (3149) primer for neo gene. MyD88+/- mice were kindly provided by Akira et. al.

Primary cell generation and cell culture
Primary mouse embryonic fibroblasts (MEFs) were prepared from E14 embryos derived from crossing of homozygous wildtype or Tollip+/- mice. Minced embryonic tissues were trypsinized and then cultured in DMEM supplemented with 10% FCS. MEFs maintained for 1-2 weeks of culture were used in these studies. Bone-marrow (BM) macrophages were isolated from femoral and tibial bone marrow cells cultured for 6-7 days in DMEM supplemented with 20% FCS and 30% supernatant from L929 cells as a source of M-CSF. Splenic DCs were isolated as described in (Didielaurent et al, 2004) and peritoneal macrophages were collected by peritoneal lavage of thioglycollate-injected mice. Spleen or thymus cell suspensions were depleted of red blood cells and cultured with indicated stimuli in RPMI supplemented with 10% FCS, 0.05mM β-mercaptoethanol and penicillin/streptomycin (Gibco).

Reagents and antibodies
A polyclonal antibody against Tollip was prepared by rabbit immunization with GST-Tollip. Antibodies anti IκBα and anti-IRAK1 were purchased from Santa Cruz. Anti-p38, anti-JNK,
anti-Phospho-IκBα were purchased from Cell Signaling. Anti-phospho specific JNK and p38 were from Biosource. Antibodies anti-phospho-Erk1/2 and anti-Tubulin, as well as recombinant murine IL-1β, LPS (E. coli O111:B4; 055:B5), CpG-ODN, PolyI:C and PGN were purchased from SIGMA. Recombinant murine IL-18 and IL-2 were purchased from MBL.

**Western Blotting**

Cells stimulated with IL-1β, LPS or PGN or transfected were lysed on ice with RIPA Buffer containing 250mM NaCl, 20 mM Tris HCl pH 7.4, 0.1% SDS, 1% NP-40, 0.5% Deoxycholic acid and Complete Protease inhibitor (Boehringer). Cell extracts were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to Hybond–P membranes (Amersham). The membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase–conjugated antibodies to rabbit or mouse IgG using the Enhanced Chemiluminesence (ECL) Western blotting system (Amersham).

**Cytokine measurements**

Measurements of IL-6, TNF-α IL-12p40, INFγ and IL-1β were performed in serially diluted samples of cell supernatants and sera from injected (LPS or IL-1β) and control mice. The measurements were performed in triplicate, using enzyme-linked immunosorbent assay (ELISA) against murine IL-6, TNF-α, IL-12p40, INFγ (R&D) according to the manufacturer’s instructions.

**Proliferation assay**

For *ex vivo* functional assays, B cells were purified from mouse spleen using anti-B220–biotin and streptavidin-coated MACS® beads (Miltenyi Biotech, purity >95%) and were stimulated in RPMI-5 for 48 hours at 5×10⁵ cells / well. Proliferation was monitored after 48 h stimulation in 96 wells plate (10⁵ cells/well) by pulsing cells for 6 h with [³²H]-thymidine. For proliferation assays with total splenocytes or thymocytes (1x10⁵ cells per well in 96-well plates) were treated or not with various stimuli as indicated in figure legends. During the final 18 h, cells were pulsed with 1mCi [³H] thymidine. Plates were collected and analyzed for [³H] thymidine incorporation using a filtermate harvester and Top Count NXT reader (Packard). Radioactive incorporation was counted by scintillation in a β-counter.

**Luciferase assay**
Primary MEFs were cotransfected using lipofectamine2000 (Invitrogen) with 500 ng of NF-κBLuc reporter plasmid obtained from V. Jongeneel (Lausanne, Switzerland) and 20 ng Renilla-luciferase transfection efficiency vector (phRLTK) (Promega). Cells were stimulated for 6 hours with 20ng/ml of murine IL-1β, 1mg/ml of LPS, 100ng/ml of TNFα. The cells were harvested and lysed and dual luciferase activity was measured in a TD-20/20 luminometer (Turner Designs) using Dual-Luciferase Reporter Assay System (Promega), according to the manufacturers’ instructions.

**Flow cytometric assay (FACS).**

The following mAbs conjugated to FITC, PE, CyChrome, or biotin were used: CD11c (HL3), MHC class II (HB11.54.3), B220 (RA3.6B2), CD86 (GL-1), CD40 (3/23), F4/80 (F4/80), all from BD PharMingen. Biotinylated Antibodies were revealed with streptavidin conjugated to CyChrome (BD PharMingen). For all staining, FcR were blocked by incubating cells with mAb anti-CD32 (2.4G2). Flow cytometry was performed using FACSCalibur and the data were analyzed by using CellQuest software.
3. Results and discussion

3.0 Objectives

Rational
Tollip was previously shown to interact with IL-1RI as well as with TLR4 and TLR2. Overexpression of Tollip partially blocked IL-1β and LPS-induced NF-κB and JNK activation, suggesting that Tollip could negatively regulate IL-1R/TLR signaling.\textsuperscript{84,113,114}

Overall objective:
To further elucidate the function of Tollip in TLRs and IL-1R signaling and more generally physiological aspects in inflammation and innate immune response. The aim of this work was to characterize the Tollip deficient mice, already available in our laboratory, using the following approaches.

1) Analyze if Tollip deficiency affects the IL-1β and TLR ligand induced NF-κB and MAPK signaling.
2) Analyze if Tollip deficiency affects IL-1β induced degradation of IRAK-1
3) Investigate the role of Tollip in IL-18, IL-1, TLR ligand induced activation of immune cells.
4) Characterize the role of Tollip in IL-1 and LPS-induced cytokine production (i.e. IL-6, TNFα) in deficient cells and in vivo, as well as in LPS-induced endotoxin tolerance.
3.1 Generation of Tollip deficient mice

Prior to the initiation of this project, Tollip deficient mice were generated in the laboratory by Kimberly Burns. To do this a targeting vector for homologous recombination was constructed by replacing exon 1 (containing the start codon) with a neomycin resistance cassette, flanked at the left side with the upstream region of the Tollip gene, and at the right side by exon 2 to exon 4 (Fig. 1.2a). Two independent and correctly targeted embryonic stem cell clones were used to generate heterozygous mice in 129sv background. The heterozygous chimeric mice were then intercrossed to obtain homozygous Tollip\(^{−/−}\) progeny. Tollip\(^{−/−}\) mice were backcrossed four times into C57BL/6 and all experiments were performed on age-matched littermates. The genotype of homozygous Tollip\(^{−/−}\) mice was verified by PCR analysis (Fig. 1.2b) and by Southern Blotting (data not shown). Western blot analysis using a mouse Tollip specific polyclonal antibody demonstrated that neither intact nor truncated Tollip protein was present in Tollip\(^{−/−}\) PBL or MEFs (Fig. 1.2c), confirming the null nature of the mutation. Homozygous Tollip\(^{−/−}\) mice were born at the expected mendelian ratio and appeared healthy, with the same life span as control littermates, indicating that Tollip is not crucial for development and survival.
Figure 1.2 Generation of Tollip deficient mice

a. Organization of the mouse Tollip gene, targeting vector and mutated allele. Filled boxes denote exons (1-6). The arrow and asterisks denote the translational initiator ATG and terminator TAA sequences, respectively. b. Analysis of genomic DNA by PCR using primers spanning the regions indicated in a, amplifying Tollip wt gene and the transgenic neo cassette. c. Western blot analysis of cellular extracts from wild type and Tollip^−/−^ MEFs, or HEK 293T cells transfected with VSV-Tollip^ΔNT^ (a.a 47–274), or full-length VSV-Tollip using antibodies (right panel), cellular extracts from peripheral blood white cells (PBLs) from wild type and Tollip^−/−^ mice, and murine lymphoid cell line EL-4 (left panel) were analyzed with indicated antibodies. To ensure equal loading the same blot was reprobed with anti tubulin antibodies.
3.2 Analysis of IκBα degradation and MAPK phosphorylation in Tollip⁻/⁻ cells

As mentioned above, Tollip has been shown to negatively regulate IL-1RI, TLR2 and TLR4 signaling\(^\text{84,113,114}\).

To facilitate the characterization of the signaling cascades potentially modulated by Tollip, bone marrow derived macrophages (BMDM) and mouse embryonic fibroblast (MEFs) were isolated from wild type and Tollip⁻/⁻ mice. The NF-κB signaling pathway downstream of the IL-1R and TLRs was examined by monitoring at degradation and phosphorylation of IκBα. Cell extracts from MEFs and BMDM stimulated with IL-1β and TLR ligands respectively (LPS, PGN and CpG). Cell extracts were analyzed by Western Blotting, revealing no difference in the level or phosphorylation of IκBα between wild type and Tollip⁻/⁻ cells following treatment with these stimuli (Fig. 1.3a and b). Moreover, IκBα which is known to be regulated by NF-κB, was re-induced normally after stimulation of Tollip⁻/⁻ cells, suggesting that NF-κB transcription activity is normally activated in the absence of Tollip. Similarly, IL-1β-, LPS- and TNFα-induced activation of a NF-κB luciferase reporter was comparable between control wild type cells and Tollip⁻/⁻ cells, indicating that Tollip is dispensable for IL-1β- and LPS-induced activation of NF-κB (Fig. 1.3c).

To characterize the role of Tollip in MAPK activation induced by IL-1R/TLRs, MAPK (p38, JNK and ERK) activation was monitored by Western Blot analysis with phosphospecific antibodies recognizing the respective activated MAPKs. MEFs were stimulated with IL-1β and BM macrophages were activated by different TLR ligands (LPS, PGN and CpG). Cell extract were analyzed at different time points by Western Blot analysis. As shown for NF-κB activation, differences were not apparent between Tollip⁻/⁻ cells and wild type cells in the phosphorylation of the MAPKs following stimulation with IL-1β, LPS, PGN and CpG.

Together, these results indicate that Tollip is dispensable for regulation of TLR-induced NF-κB and MAPK signaling pathways in BM macrophages and MEFs.
Figure 1.3. Analysis of IL-1β, TLR ligands-triggered signaling cascades in Tollip<sup>−/−</sup> cells
Primary MEFs (a) or bone marrow-derived macrophages (b) from wild-type and Tollip<sup>−/−</sup> mice were stimulated with LPS (1 µg/ml), PGN (10 µg/ml), CpG (100µg/ml) or IL-1β (50 ng/ml) for the indicated times. Cell extract were prepared and analyzed by Western Blot with various antibodies as indicated. c. MEFs were transfected with 500 ng pLuc NF-κB reporter, and 20 ng of phRLTK (Renilla Luciferase). The cells were stimulated for 6 hr with IL-1β (20 ng/ml), LPS (1 µg/ml) or TNFα (100 ng/ml), lysed and luciferase activities were determined using the Dual-Luciferase Reporter Assay System. Values shown are averages of data obtained in three transfection experiments.
3.3 Normal IRAK1 degradation in Tollip\(^{-}\) cells

Tollip binds to IRAK-1 in unstimulated cells and both proteins are recruited with similar kinetics to the IL-1R complex. As Tollip binding suppresses the kinase activity of IRAK-1, it has been suggested that Tollip controls IRAK-1 recruitment to the IL-1 receptor and keep IRAK-1 in an off-state prior to receptor triggering \(^{84,113}\). After IL-1\(\beta\) stimulation and recruitment to the IL-1RI complex, IRAK-1 is rapidly autophosphorylated, triggering its proteasomal degradation. Tollip deficiency therefore would presumably affect IL-1\(\beta\)-dependent degradation of IRAK-1.

To address this question, wild type, Tollip\(^{-}\) and MyD88\(^{-}\) MEFs were stimulated with IL-1\(\beta\) (Fig. 1.4). Western Blot analysis revealed that IRAK-1 was degraded with similar efficiency and kinetics in both cell types following stimulation with IL-1\(\beta\) at 20 ng/ml (Fig. 1.4a). Further, even at suboptimal concentrations of IL-1\(\beta\), degradation of IRAK-1 and I\(\kappa\)B\(\alpha\) occurred in both wild type and Tollip\(^{-}\) cells (Fig. 1.4b). MyD88 is essential for the IL-1R/TLR signaling and the degradation of IRAK-1 and I\(\kappa\)B\(\alpha\). As expected, no I\(\kappa\)B\(\alpha\) and IRAK-1 degradation were detected under the same conditions in MyD88\(^{-}\) MEFs (Fig. 1.4b).

Altogether, these results suggest that Tollip/IRAK-1 interaction is neither essential for recruitment to IL-1RI/IL-1RACP nor for the activation/degradation of IRAK-1.

![Figure 1.4](image.png)

**Figure 1.4. IRAK-1 degradation occurs normally in Tollip\(^{-}\) cells.**

a. Primary MEFs from Tollip\(^{-}\) or wild type mice were stimulated with IL-1\(\beta\) (20 ng/ml) for the indicated times. Cell lysates were prepared and analyzed by Western Blot with anti-IRAK-1 or anti-I\(\kappa\)B\(\alpha\) antibodies. b. wild-type and Tollip\(^{-}\), or MyD88\(^{-}\) MEFs were stimulated with the indicated concentrations of IL-1\(\beta\) for 15 min. Cell lysates were prepared and immunoblotted with various antibodies as indicated. As a loading control in these experiments, the same membrane was reprobed with antibodies against tubulin. NT: not treated
3.3 Activation of immune effectors cells in Tollip\(^{-/-}\) mice

IL-1R and TLRs are known to induce the activation and proliferation of immune cells such as lymphocytes and dendritic cells (DC). As deregulated signaling may affect the cellular composition of immune organs, the cellular composition of the thymus, spleen, lymph node and bone marrow was evaluated and found to be comparable between Tollip\(^{-/-}\) and wild type mice (data not shown). This indicates that Tollip is dispensable for the development of immune cells and organs.

3.3.1 Normal TLR-induced activation and proliferation in Tollip\(^{-/-}\) cells

TLR-induced proliferation of splenic B cells is known to be MyD88 dependent\(^{77,78}\). Proliferation assays were carried out with wild type, Tollip\(^{-/-}\) and MyD88\(^{-/-}\) total splenocytes treated with different doses of LPS. LPS-induced proliferation of Tollip\(^{-/-}\) splenocytes was comparable with wild type cells, in contrast to MyD88\(^{-/-}\) splenocytes whose proliferation was strongly impaired (Fig. 1.5a). In addition, LPS- and CpG-induced upregulation of MHCII expression on purified B cells were monitored by flow cytometry. This experiment showed that upregulation of MHC class II on purified B cell was also not affected by loss of Tollip in contrast to MyD88 deficiency, suggesting that Tollip is not essential for B-cell activation (Fig. 1.5b).

TLR-induced dendritic cell (DC) activation including the upregulation of costimulatory molecules such as CD86 and CD40 is essential for the priming of antigen-specific T cells. To determine whether the Tollip deficiency would influence TLR-induced DC activation, Tollip\(^{-/-}\) and wild type mice were injected with LPS or CpG. Six hours later, the activation of CD11c\(^+\) splenic DC was monitored by FACS analysis. CD86 and CD40 levels were similarly increased on CD11c\(^+\) splenic DC following LPS injection of both Tollip\(^{-/-}\) and wild type mice (Fig. 4c). Similar results were obtained when mice were injected with TLR9 and TLR5 ligands (respectively CpG and Flagellin) (data not shown). Accordingly, activation of BMDC by these same ligands confirmed that Tollip does not play a role in TLR-induced DC activation (Jean-Claude Sirard, personal communication).
Figure 1.5. Tollip-independent Activation of DC and splenocytes by TLRs ligand (LPS, CpG) stimulation.
a. Total Splenocytes were incubated with LPS or CpG at the indicated concentration and proliferation was monitored 48 h later by pulsing the cells overnight with \(^{[3]}\)H-thymidine. b. Splenic B cells were isolated using B220\(^+\) MACS beads and incubated with 5 µg/ml of LPS or CpG for 48h. Surface expression of MHCII was then monitored by flow cytometry. c. Tollip\(^+/-\), myd88\(^+/-\) and wild type mice were injected i.v. with 1 µg of LPS and 6 hours later splenic DCs were analyzed by flow cytometry for the indicated activation markers (CD86 and CD40). Histograms represent data for F4/80\(^{low}\)/CD11c\(^+\) gated cells.
3.3.2 IL-1 and IL-18 induced activation and proliferation in Tollip<sup>−/−</sup> cells

As Tollip also interacts with the cytoplasmic tail of IL-18R in co-expression experiments, we assayed the effect of Tollip deficiency on the IL-18 signaling. IL-18 is known to induce lymphocyte proliferation and INF-γ production<sup>117−119</sup>. To address this question we performed experiments with splenocytes isolated from wild type control, Myd88<sup>−/−</sup> and Tollip<sup>−/−</sup> mice. Splenocytes were stimulated with mouse IL-18 and also IL-12, in order to enhance the response, and then were analyzed for IFN-γ production by ELISA (Fig. 1.6a, upper panel) and for induction of proliferation (Fig. 1.6a, lower panel). We observed that proliferation and IFN-γ production was completely abrogated in Myd88<sup>−/−</sup> cells as expected<sup>77,78</sup>; however no significant differences were seen between Tollip<sup>−/−</sup> and wild type cells (Fig. 1.6a).

IL-1β is known to be a co-stimulant for T cell proliferation and activation<sup>120,121</sup>. To determine if Tollip affects the costimulatory role of IL-1β during TCR activation and IL-2 stimulation, we assessed ex vivo proliferation assays with isolated wild type and Tollip<sup>−/−</sup> thymocytes (Fig. 1.6b). Wild type and Tollip<sup>−/−</sup> thymocytes showed similar enhanced proliferation when cultured with the TCR crosslinker concanavalin A (ConA) or IL-2 in the presence of IL-1β. Figure 5b shows an experiment in which Tollip<sup>−/−</sup> cells were found to have slightly reduced proliferation following IL-1β/IL-2 stimulation relative to wild type cells, but this was not confirmed in other similar experiments.
Figure 1.6. Tollip–independent IL-1β and IL-18 induced activation and proliferation of lymphocytes.
a. Splenocytes were incubated in triplicate in medium containing 10 ng/ml IL-12, 20 ng/ml IL-18 or both. The production of INF-γ in the supernatant was measured 72h later by ELISA (upper panel) or for proliferation by [3H]-thymidine incorporation (lower panel). b. Thymocytes were incubated in medium containing 20 U/ml IL-2 or ConA with or without 10 ng/ml IL-1β for 3 days. The cell proliferation was measured by pulsing the cells overnight with [3H]-thymidine. Data are average of quadruplicates and are representative of 3 independent experiments.
3.4 Tollip−/− mice produce less cytokine following IL-1β and LPS stimulation

IL-1/TLR signaling activates several transcription factors regulating the expression of numerous genes, including proinflammatory mediators. To further evaluate the role of Tollip deficiency, we measured the production of cytokine (IL-6, TNF-α, IL-12p40) upon LPS and IL-1β stimulation in Tollip−/− and wild type mice and primary cells (Fig. 1.7 and 1.8).

Wild type and Tollip−/− cells were stimulated with low doses of IL-1β and analyzed by ELISA for IL-6 production. Unexpectedly, Tollip−/− cells were found to produce less IL-6 than wild type cells (Fig. 1.7a). Similarly, it was also found that peritoneal macrophages derived from Tollip−/− mice produced less IL-6 following treatment with IL-1β (Fig. 1.7b). Dose-dependent reduction in IL-6 and TNF-α production was also observed in peritoneal macrophages stimulated with LPS (Fig. 1.7b). In summary, we observed a decreased cytokine production in Tollip deficient cells upon stimulation at low concentrations of IL-1β. In contrast with the previous studies showing Tollip as an inhibitor of IL-1β and LPS pathways, these data suggest that Tollip has a role in the fine tuning of the IL-1β and LPS induced signaling.

Interestingly, IRAK-1−/− macrophages have a similar phenotype, since LPS-induced TNF-α secretion is impaired at low doses, but not high doses of LPS 122.

To determine the effect of Tollip deficiency in vivo, inflammatory cytokine production (i.e. IL-6, TNF-α) was measured in the sera of wild type and Tollip−/− mice following intravenous (i.v.) injection with IL-1β or LPS. Tollip−/− mice produced significantly less IL-6 than their wild-type littermates following i.v. injections of IL-1β (1 µg) or low doses of LPS (50 µg/kg) (Fig. 1.8a). However, following administration of higher lethal dose of LPS (40 mg/kg), production of IL-6, TNF-α, as well as IL-12p40 was similar in wild type and Tollip−/− (Fig. 1.8b). Correspondingly, no difference in susceptibility to LPS-induced septic shock were detected; Tollip−/− mice died as rapidly as their wild-type littermates.

These results together indicate that Tollip could have an important role in the fine tuning of signaling pathways inducing the production of proinflammatory cytokines, at concentrations of IL-1β and LPS that are likely physiologically relevant (i.e. following infection, inflammation).
Figure 1.7. Reduced Cytokine production in Tollip\(^{-/-}\) cells at low doses of IL-1\(\beta\) and LPS stimulation.
a. Primary MEFs (a) and peritoneal macrophages (b) from Tollip\(^{-/-}\) or wild type mice were respectively stimulated with 20ng/ml of IL-1\(\beta\) or indicated concentration of IL-1\(\beta\) (b). The production of IL-6 in the supernatant was measured by ELISA 24 h (a) or 9h (b) later. b. Peritoneal macrophages (b) from Tollip\(^{-/-}\) or wild type mice were stimulated with indicated amounts of LPS. The production of IL-6 and TNF\(\alpha\) in the supernatant was measured after 9 hours by ELISA.
Figure 1.8. Reduced cytokines in the sera after low dose of IL-1 and LPS stimulation in Tollip−/− mice.

a. Tollip−/− or wild-type mice were injected i.v. with 1 µg of IL-1β (5 mice/group) or 100µg/kg of LPS (3 mice/group) and the production of IL-6 in the serum was measured at 2 h by ELISA. b. Tollip−/− or wild-type mice were injected i.p. with a lethal dose (40mg/kg body weight) of LPS (E. coli 0111:B4) to induce septic shock. The serum concentrations of IL-6, TNFα, IL-12p40 for all mice/group (n=9) were measured by ELISA 3 h after injection and data shown as average for each group. Animals were then monitored during 100 hours postinjection for death or moribund state over the test period.
4. Conclusions

In summary, we found that Tollip is not essential for the TLR4- and IL-1R-induced activation of the NF-κB and MAPK pathways as detected by Western blotting or luciferase assays. Immune cells activation induced by TLR ligand and IL-1 or IL-18 were also not affected by Tollip deficiency. The foreseen role of Tollip, i.e. as a negative regulator for these signaling pathways, was therefore not confirmed in mice. However, Tollip appears to be necessary for the production of cytokine after stimulation with physiological doses of IL-1 or endotoxin. This suggests that Tollip is implicated in the fine tuning of the signaling events leading to cytokine production.

5. Discussion and perspective

**Tollip and the TLR/IL-1R classical signaling pathways**

The analysis of mice deficient in various other components of the TLR and IL-1R signaling cascades (i.e MyD88, TRAF6, IRAKs) often have revealed their implication (inhibition, activation) in signaling event of the cascade and consequently in cytokine production (TNFα, IL-6, IL-1β). In view of previous overexpression studies in cells line that defined Tollip as an inhibitor of TLR and IL-1R signaling pathway\(^{84,113,114}\), an increase in signaling and cytokine production was expected. Surprisingly, the preliminary characterization of the Tollip deficient cells showed between these and control cells no apparent difference in biochemical events of the NF-κB and MAPK cascade (phosphorylation and degradation events). It is therefore possible that Tollip has, indeed, no role in the initial events of these signaling pathways. However, we cannot exclude the presence of other proteins which could be redundant with Tollip, replacing its function in NF-κB and MAPK signaling. Possible candidate proteins that might substitute for Tollip include like the CUE-containing TAB2 or TAB3\(^{123-126}\). One could also argue that a slight difference in the activation of the NF-κB and MAPK pathways, resulting in partial reduction of cytokine production, is not detectable by the methods used in this study (Western blotting or luciferase assays). Tollip deficient cells have reduced cytokine production only when stimulated with low doses of LPS and IL-1β. However, all experiments aimed at measuring the biochemical events of these pathways have been performed at high dose of stimuli, which may override the signaling system, as observed when high dose of LPS is used in vivo (Fig 1.1 and Fig. 1.2). To confirm this statement, these experiments should be repeated more carefully using lower doses of stimuli. We have also to consider the possibility that kinetic and amount of production of
certain late cytokines (i.e. IL-6, IL-12) after stimulation/injection with TLRs ligand or IL-1β could be altered or delayed, as results of reduced induction of others early cytokine (i.e. TNFα). To test this hypothesis several others cytokines production should be followed a different kinetics with different doses of IL-1 β, LPS and CpG in Tollip deficient mice.

Strikingly, the phenotype of Tollip knock-out mice is very similar to the phenotype of the IRAK-1 deficient mice. Tollip was shown to interact at the molecular level with IRAK-1 in the TLR/IL-1R signaling cascade, suggesting that Tollip and IRAK-1 functions could be related due to their interaction, and be influenced reciprocally. Tollip could indeed modulate IRAK-1 function, affecting partially the interaction of IRAK-1 with other proteins such as TRAF6 and/or phosphorylation of IRAK-1 during signaling. Tollip deficiency could also lead to a deregulation of IRAK-1 function, which would explain the similar phenotype observed between the Tollip and IRAK-1 deficient mice. To further investigate if Tollip and IRAK-1 have cooperative function, it would be interesting to analyze the phenotype of the double knockout IRAK-1/Tollip mice, eventually observing attenuated or increased response in these mice compared with IRAK-1 deficient mice.

**Tollip and others signaling pathways**

The reduction of IL-6 and TNF-α observed in Tollip deficient mice could also be explained by decreased activation of pathways other than those activating NF-κB and AP-1. Others IL-1R /TLR pathways such as Interferon Regulatory Factor (IRF) or the PI3K/Akt pathways could be mediated by the IRAK-1/TRAF6/Tollip platform and are indeed important for cytokine production. The IRFs and in particular IRF7, IRF5 and IRF3 are required for the induction of IFNα/IFNβ by TLR ligands, but also that NF-κB and IRF signaling cascades use similar signaling molecules, such TRAF6 and myd88 and their activation is inter-dependent. In fact, it was shown that IRF7 is crucial for TLR (i.e. TLR3, TLR4, TLR9) for IFNα/IFNβ production in a MyD88-independent manner, in an other hand, IRF5 in an MyD88/TRAF6-dependent manner activate also pro-inflammatory cytokines (i.e. IL-12, IL-6), as showed in IRF5 knockout mice, where INFα production is normal, and TNF-α, IL-6 production is impaired.

Interestingly, it was recently shown that in some cases IL-1β induces IFN responsive genes via IRF3, suggesting an involvement of these transcription factors in IL-1 pathway. It is therefore possible that Tollip may also be involved activation of IRFs, and in particular IRF3 and IRF5, as its deficiency partially affectsthe production of cytokines.
Togther with our results showing that Tollip binds TRAF6 in signaling complex, suggest that Tollip could modulate NF-κB, and also IRF pathways. To assess this hypothesis, the IRF phosphorylation as well as the INFα/β production should be measured in Tollip-deficient cells stimulated with IL-1β, CpG or LPS.

Another pathway activated by TLRs and IL-1R in an IRAK-1-dependent manner is the PI3K/akt pathway. PI3Kinase directly interacts with IL-1R. The activation of PI3K/Akt affects the activation of NF-κB and is required for cytokine production. This latter study together with features of the Tollip protein supports the hypothesis that Tollip could regulate PI3K via its C2 domain. Indeed, Tollip interacts with isoforms of PI3K subunits (p110/p85) in overexpression experiments (Brian Brissoni, unpublished data) eventually via the C2 domains. To study the role of Tollip in PI3K activation Tollip deficient cells and wild type should be compared for IL-1β/LPS-induced Akt phosphorylation and activation of down stream events of PI3K. In addition to study the role of Tollip on PI3K activity, and its influence on and NF-κB and MAPK, as well cytokines production, Tollip deficient or wild type MEFS could be stimulated with or without a PI3K inhibitor (Wortmanin), to see reduction or enhancement of the response.

**Tollip levels and signaling regulation**

As shown by previous studies Tollip is induced by LPS, and Tollip upregulation inhibits NF-κB signaling. However, we have shown that Tollip is not essential for NF-κB and MAPK signaling activation but instead modulates the signaling. Suggesting that low levels of Tollip expression results in optimal response to proinflammatory signals or pathogens, whereas at high levels of expression of Tollip could inhibit signaling resulting in hyporesponsive cells. To further characterize the dual role of Tollip resulting from its upregulation during restimulation tolerance it would be interesting to compare the responses of both Tollip deficient mice and Tollip transgenic mice to TLRs ligand, IL-1β and in vivo challenge with pathogens.

**Tollip deficiency and in vivo responses to pathogens**

The production of pro-inflammatory cytokine is crucial to initiate and sustain anti-microbial defense mechanisms. Regulation of cytokine production allows the efficient clearance of pathogens without causing by-stander tissue damage (immunopathology). Altered cytokine production in Tollip deficient animals may impair their ability to clear infections, in particular because most of the defense mechanisms induced by pathogens rely
on the TLR and IL-1β pathways. It will therefore be important to challenge Tollip deficient mice with various pathogens including bacteria such as *Staphylococcus aureus* or *Salmonella* by systemic or mucosal infection. Also a model of viral infection should be performed (Influenza for example), as this is a context where the INF response plays a crucial role. It would be relevant to monitor for example: cytokine production, ability to clear pathogens (bacterial and viral counts), general health status (weight loss, cachexia).

Interestingly, Tollip was recently reported to be involved in hyporesponsiveness to LPS stimulation and commensal bacteria in the gut [116]. Tollip mRNA and protein expression is upregulated upon stimulation and may therefore participate in the hyporesponsiveness of intestine epithelial cells (IEC). Probably upregulation of Tollip activates its inhibitory role in the signaling, resulting in a significantly decreased proinflammatory response.

In the gut, IECs are continuously exposed to stimulation by the natural bacteria microflora that produce PAMPs including TLR ligands [135]. In order to avoid the negative effects of constitutive inflammation, the cells in the gut are generally hyporesponsive. The endotoxin tolerance develops in the cells following a second or continuous challenge with a TLR ligand or bacteria, inducing a blockade in the NF-κB and MAPK signaling pathways [136,137]. The study by Otte and colleagues implies that Tollip deficiency could affect the homeostasis in the gut, affecting the main structure of the intestinal tissues [116]. Alteration of the gut homeostasis could lead to inflammation syndromes such as bowel disease [138,139]. Therefore, even if Tollip knockout mice appear healthy, careful histological examination of their intestinal tissues should be performed. To further characterize if Tollip deficiency compromises TLR-induced tolerance or innate response in the intestine, several models of inflammation and mucosal infections should be performed on Tollip deficient mice. A model for tissue damage and inflammation in the intestines is the dextran sulfate sodium (DSS)-induced acute colitis model. The mice are orally treated with DSS and tested for colonic mucosal injury and inflammation (body weight loss, biochemical and histological analysis). Model of mucosal infection such as Listeria or Salmonella should also be performed in Tollip deficient mice and the mice monitored for cytokine production (i.e CXCL1), lethality and intestinal tissue structures.

Tollip’s endocytic role and TLR/IL-1R signaling
Tollip could influence the TLR/IL1R signaling by its functions as an endocytic adaptor in the endocytosis of IL-1R and eventually TLRs (see chapter II). It is therefore possible that cytokine production in the absence of Tollip is altered due to: deregulated endocytosis of the receptors (in the case of IL-1R, see chapter II), or abnormal endosomal localization of the signaling complex inside the cells.

Tollip deficiency could affect the endocytic pathway deregulating the internalization/recycling rate of the receptors and reducing the level of receptors at cell surface. This could influence the strength and duration of signal and consequently cytokine production.

The localization of the signaling complex associated with a given receptor and signal was shown be is important for spatio-temporal regulation of the signaling and for its specificity. A recent study for TLR9 suggests that localization of the complex is a crucial event that determines which cytokine is produced. Tollip present in the endosomal compartment may participate as endocytic adapter in the docking of signaling complex at the endosome for specific signals. Whether, if Tollip participate in this mechanism should be further explored by experiment of microscopy colocalization of signaling proteins (i.e MyD88, TRAF6) in Tollip deficient cells (Plasmacytoid DC) of internalized of signaling complex combined with specific cytokine production (INF, IL-6, IL-12).
Chapter II:
Regulation of IL-1R endocytosis by Tollip
and its CUE domain
1. Introduction

1.1 Ubiquitination and ubiquitin functions

1.1.1 Ubiquitin

The fundamental role of ubiquitin in eukaryotic organisms is known since the late 1970s when ubiquitin was discovered in yeast and evolved eukaryotes. Ubiquitin finds its name in the fact that it is ubiquitously expressed in all eukaryotes. Ubiquitin is a small globular protein of 76 amino acids (7-8 kDa) highly conserved through all eukaryotic organisms (more than 97% of identity between yeast and human ubiquitin). It has the property to bind covalently to other proteins. Ubiquitin and ubiquitination appear to be essential for the regulation of a multitude of vital processes for cell biology.

1.1.2 The ubiquitination mechanism

Protein ubiquitination is a multistep process that requires the sequential action of three types of enzymes, the ubiquitin-Activating Enzyme (E1), the ubiquitin-Conjugating Enzymes (E2s) and the ubiquitin-Ligases (E3s). Only one single E1 was described in mammals and is present in two different isoforms. There are several E2 enzymes, and more E3 ligases than E2 enzymes, leading to a pyramidal combination of enzymes and therefore increased specificity for the substrate in the cell. The first step of the ubiquitination process is the recognition and binding of ubiquitin to the ubiquitin-Activating Enzyme (E1). There is the formation of a thioester bond, in an ATP-dependent manner, between the C-terminal glycine residue of the ubiquitin and a single conserved cystein of the E1 enzyme. Ubiquitin is then transferred from the E1 through trans-thyolation to one of the several E2 enzymes on a UBC domain (Ubiquitin Binding Domain). There are 13 E2/Ubc enzymes in yeast, called Ubecs, very homologous to the 25 found in mammals. The UBCH/Ubc contains a C-terminal or N-terminal extension conferring the capacity to bind specifically an E3 ligase. There are a large number of these E3 enzymes that provide the substrate specificity, recognizing the ubiquitination-targeted proteins. There are at least two types of E3 enzymes characterized by the presence of either a RING domain or an HECT domain (Fig. 1). The RING E3 ligases catalyze the transfer of ubiquitin from the E2 to the substrate, whereas the HECT E3 ligases directly bind the ubiquitin and transfer the bound ubiquitin to a lysine residue on the target protein. The E1/E2/E3 ubiquitination machinery adds the first ubiquitin moiety, binding the carboxyl group of the N-terminal glycine of ubiquitin to the ε–amino group of lysine of the target protein. In the same way the E1/E2/E3 ubiquitination machinery adds additional
ubiquitin moieties to form ubiquitin polychains (Fig. 2.1).

![Figure 2.1. Schematic representation of the ubiquitination machinery.](image)

**1.1.2 Different forms of ubiquitination and their functions**

Ubiquitin is present in the cells as a free form or conjugated to other proteins as monoubiquitin or as polyubiquitin chain. The monoubiquitin or polyubiquitin modification of proteins were shown to regulate different cellular processes. Seven lysine residues are present in ubiquitin (K6, K11, K27, K29, K33, K48 and K63) which can be used as acceptor sites for the addition of ubiquitin moieties to form an ubiquitin polychains. In vitro all seven may act as donors to form an ubiquitin polychain. However, only polychains with Ub-Ub linkage through K29, K48 and K63 have been detected in vivo.

Proteins conjugated with K48 polychains, containing at least four moieties, are targeted for proteolytic degradation mediated by the 26S proteasome. The 26S proteasome is a large cylinder-shaped multimeric complex composed of more than 30 subunits with multiple proteolytically active sites. Ubiquitin polychains are recognized by the proteasome and detached from the target protein for the hydrolysis to free ubiquitin, while the proteins is unfolded and degraded at the proteolytic active site of the 26S proteasome. So far ubiquitin targeted degradation of key metabolic or regulatory proteins has been shown to be govern multiple cellular processes, such as eliminating key metabolic or regulatory proteins in response to signals.
During the last years we gained new insight in others ubiquitin functions that are proteasome-independent. In particular, K63-linked polychains are involved in a variety of other cellular processes. Recently it was shown that K63-linked polychain modifications are important in kinase activation and signal transduction. TRAF6 is modified by K63 polychains, in a stimulus dependent manner, inducing the activation of TAK1, an upstream activator of IKK and MKK responsible for NF-κB and MAPK activation, respectively. Similarly, also TRAF2 is modified by K63 polychains during TNF signaling. K63 ubiquitin polychains were also found to modify and regulate protein involved in regulation of DNA Repair and receptor endocytosis.

Monoubiquitination of proteins in eukaryotes targets proteins for lysosomal/vacuolar degradation, regulation of DNA repair, and can act as an internalization and sorting in endocytic and biosynthetic pathways.
1.2 Ubiquitin signal in the endocytic pathway

The appropriate cellular response depends not only from the nature of the signal, but also on the signaling mechanism. This means that the duration and strength of the signal need to be regulated in order to ensure cellular homeostasis and to prevent uncontrolled cell transformation or proliferation, causing disorders and diseases.

One mechanism by which eukaryote cell can downregulate signaling is by modulating the surface levels of a receptor in response to a stimulus, through the endocytosis. Numerous surface receptors, such as growth factor receptors, ion channels, cytokines and antigen receptors, are internalized by endocytosis for degradation\textsuperscript{157}.

Ubiquitination has been recently shown to play a key role in activation and regulation of the endocytic pathway. Ubiquitination acts as a signal for signal-dependent or constitutive internalization of surface receptors into the endosome, as a tag for internalization of cargo proteins or by regulating the functions of proteins involved in the endocytic machinery (endocytic adaptors)\textsuperscript{156}.

1.2.1 The endocytosis and Endosome structure

The different mechanisms that are collectively termed endocytosis serve many important cellular functions including the uptake of liquids or particles from the surrounding environment, as well as internalization of lipids and proteins from the plasma membrane. Endocytosis can be broadly divided in phagocytosis, for ingestion of large particles and pinocytosis for liquids (or cell drinking)\textsuperscript{158,159}.

Endocytosis can be constitutive or triggered by different mechanisms, such as recognition of external particles by specific receptors during phagocytosis, or internalization signals by membrane receptors. Endocytic processes lead to budding of the plasma membrane and formation of lipid-bilayer vesicles delivered inside the cells into an ensemble of vesicular specialized organelles called endosomes (Fig. 2.2). The endosome is an elaborate network of intracellular vesicular organelles regulating endocytic, biosynthetic and secretory pathways, including downregulation of surface receptor signaling and internalization into vacuolar/lysosomal compartments, recycling, targeting newly synthesized proteins for external delivery (secretion), antigen internalization and processing.

The endosomes include different vesicular organelles such as early and late endosomal compartments, the lysosome/vacuolar compartments, the \textit{trans}-Golgi network, and all kinds of vesicles (i.e. secretory vesicles, phagocytic vesicles). Each endosomal compartments has a precise function and is characterized by the presence of specific membrane protein and lipids.
composition \textsuperscript{160}. All the compartments of the endosome are interconnected by trafficking vesicles formed by the budding of a portion of the organelle membrane and fusion with the other organelles.

The beginning of the endocytic pathway is the formation of primary vesicles from plasma membrane triggered by different internal or environmental signals. Primary vesicles formation is promoted by protein coated-pits that pinches off the plasma membrane leading first to invagination of the membrane and then to budding. Coated-vesicle formation and internalization from the plasma membrane are promoted by coat protein (i.e. caveolin and clathrin), adaptor protein (i.e. AP-2), motor proteins (i.e. the GTPase dynamin) and is helped by special lipids enrichment inducing the membrane curvature for invagination (i.e. cholesterol, glycosphingolipids).

Primary endocytic vesicles are uncoated and delivered to the peripheral early-endosome. The Early endosome dynamics are regulated by multiple effectors, all typical protein markers of the early-endosome. These effectors are the GTPase Rab5 that recruits EEA1 and Syntaxins, the PI-3 Kinase and PI3P-binding proteins \textsuperscript{161}. The early-endosome is the crossway of divergent exit pathways leading to recycling of cargo back to the cell surface and trafficking of cargo to the late-endosome. Ubiquitinated cargo (i.e. EGFR) trafficking and sorting from early to late endosome are mediated by ESCRTI to III complexes (Endosomal Sorting Complex Required for Transport) and their binding proteins, such as Tsg101. In addition, other proteins act upstream of ESCRTI for cargo trafficking, such as Hrs/Vps27 that recognizes ubiquitinated cargo \textsuperscript{162-165}.

The late endosome also referred as multivesicular bodies (MVBs) is distinguishable from early-endosome by a number of characteristics such as localization, reduced acidic pH and morphology. The late-endosome is also characterized by several markers. For example it contain within the internal membrane layer the non-degradable lipids, such as lysobisphosphatidic acid (LBPA) \textsuperscript{166} and attached to this external membrane layer the glycoproteins LAMP1 and 2 \textsuperscript{167}. Other proteins are present in the late endosome for normal trafficking to the lysosome, like Rab7 \textsuperscript{161,168}. From the late-endosome cargo proteins can be sorted to lysosomal compartments or recycled back to early-endosome, Trans-Gogi Network (TGN) or plasma membrane. The fusion of mature Late-endosome/MVB with the lysosome results in proteolysis of the lipid and protein contents of these vesicles (Fig. 2). (reviewed in \textsuperscript{157,160,165,169}).
Figure 2.2. Schematic model of the endosome: organelle structures, cellular trafficking and features.
(1) Ligand binding causes the clathrin coated pit formation. (2) Phagocytic and endocytic primary vesicles are internalized. (3) The sorting endosome traffics vesicles to the Early-Endosome. The early vesicles are involved in trafficking Late-endosome (4a) and with the trans-Golgi Network (TGN) (4b). Early-Endosome vesicles can return to the plasma membrane for receptor recycling and secretion (5): The Late Endosome (MVB) is the way for trafficking from TGN (6) to lysosome compartment for proteolytic degradation (7).
1.2.2 Ubiquitin-dependent internalization and trafficking in the endocytic pathway

In eukaryotes endocytic and biosynthetic pathways converge at endosomes, which regulate the delivery of proteins to the membrane or into the lysosome/vacuole respectively\(^{165}\). Ubiquitination regulates the endocytic processes in the cell in at least three different mechanisms: (a) Ubiquitin can serve as a sorting signal attached to membrane receptors for internalization and direct their movement between different endosomal compartments; (b) Ubiquitin can modify the activity of the endocytic proteins during vesicle formation, trafficking and sorting; (c) Ubiquitination regulates the lysosomal and proteosomal degradation of these membrane proteins\(^{156,170}\).

1.2.2.1 Ubiquitination of transmembrane receptors in yeast and mammals

The first observation of transmembrane receptor ubiquitination was made in the mid-1980s when several Growth Factor Receptors were shown to be ubiquitinated (i.e. GHR, β-PDGFR). The PDGF and GHR were found to be ubiquitinated at their cytoplasmic tails and were subsequently shown to undergo ligand-stimulated ubiquitination\(^{171,172}\). Later, multiple yeast and mammalian transmembrane receptors were also shown to be ubiquitinated. As the numbers of ubiquitinated receptors constantly expand. I have given a list of examples of ubiquitinated transmembrane receptors in Table 2.1. For the majority of these receptors it has been shown that ligand binding triggers internalization and degradation\(^{156,173,174}\).
### Table 2.1. Selected ubiquitinated transmembrane proteins

<table>
<thead>
<tr>
<th>Transmembrane proteins</th>
<th>Functions</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Factor receptor (Ste2, Ste3)</td>
<td>Response to mating hormone</td>
<td>Yeast</td>
<td>Hicke et al., 1999</td>
</tr>
<tr>
<td>α-Factor Transporter (Ste6)</td>
<td>Response to mating hormone</td>
<td>Yeast</td>
<td>Roth &amp; Davis et al., 2000</td>
</tr>
<tr>
<td>Uracil Permease (Fur4)</td>
<td>Nutrient uptake</td>
<td>Yeast</td>
<td>Kölling &amp; Losko et al., 1997</td>
</tr>
<tr>
<td>General Amino Acid Permease (Gap1)</td>
<td>Amino acid uptake</td>
<td>Yeast</td>
<td>Rotin et al., 2000</td>
</tr>
<tr>
<td>Triptophan permease (Tat2)</td>
<td>Amino acid uptake</td>
<td>Yeast</td>
<td>Beck et al., 1999</td>
</tr>
</tbody>
</table>

**Signaling**

- **GHR**
  - Chemokine receptor
  - Species: Mammalians
  - Reference: Strus & Govers et al., 1999

- **EGFR**
  - RTK signal transduction
  - Species: Mammalians
  - Reference: Shtiegman et al., 2000

- **PDGFR**
  - RTK signal transduction
  - Species: Mammalians
  - Reference: Lee et al., 1999

- **TGFβ**
  - Development
  - Species: Mammalians
  - Reference: Kvasak et al., 1999

**Immunity**

- **Pre-TCR**
  - Immunoglobulin superfamily
  - Species: Mammalians
  - Reference: Panigada et al., 2002

- **TCR CD3**
  - Immune recognition
  - Species: Mammalians
  - Reference: Cienciarelli et al., 1992

- **CD4**
  - TCR associated
  - Species: Mammalians
  - Reference: Kvasak et al., 1999

- **MHC Class I**
  - Antigen presentation
  - Species: Mammalians
  - Reference: Mansouri et al., 2003

- **Fcyreceptor II**
  - Immunoglobulin superfamily, phagocytosis
  - Species: Mammalians
  - Reference: Booth et al., 2002

**Others**

- **CXCR4**
  - Chemokine receptor
  - Species: Mammalians
  - Reference: Marchese et al., 1999

- **β-adrenergic Receptor**
  - Muscle signaling
  - Species: Mammalians
  - Reference: Shenoy et al., 2002

- **Notch**
  - Development
  - Species: Mammalians
  - Reference: Qiu et al., 2000

- **ENaC**
  - Na⁺ Ion Channel
  - Species: Mammalians
  - Reference: Rotin et al., 2000
1.2.2.2 Ubiquitin as internalization and sorting signal for cargo proteins into the endosome

The first link between ubiquitin and internalization was observed in yeast, where receptor Ste6 accumulates in ubiquitin-deficient strains \(^{175}\). Additional information on ubiquitin as a signal triggering internalization came from the combined effort of biochemical and genetics studies showing that ubiquitination promotes, both constitutive and signal triggered, internalization and lysosomal degradation of yeast GPCRs Ste2 and Ste3; while this was impaired or reduced in yeast strains deficient in genes encoding for ubiquitinating enzymes (i.e. E2 and E3 ligases) \(^{176}\). In addition, mutations of lysine residues identified as ubiquitination sites in cytoplasmic domains of the receptors inhibits their internalization \(^{156,170}\).

The final proof for the role of ubiquitin as internalization signal, came from experiments with an ubiquitin fusion chimera of yeast and human receptors. The fusion of ubiquitin to the cytoplasmic tail is sufficient for their internalization \(^{177}\). Conjugation of monoubiquitin to a single lysine residue on the intracellular tail of membrane receptors is sufficient for rapid internalization of several transmembrane proteins in yeast and mammalian cells \(^{178-180}\). For many receptors, such as EGFR (Epithelial Growth Factor Receptors), ligand binding induces monoubiquitination that acts as a regulated internalization signal that does not require additional sequences in the cargo protein to function \(^{179,181-183}\). This ubiquitin signals on the receptor induce the recruitment of proteins involved in the endocytic pathways, the endocytic adaptors. These endocytic adaptors often contains ubiquitin-binding domain that are responsible for the recognition of receptor ubiquitination.

In addition ubiquitination acts in the endosome as internalization signal of cargo proteins, as key element in the regulation of the endocytic pathway. The first hint that ubiquitin play a role in endocytic pathways came from studies on the trafficking of EGFR that is internalized ligand-dependent manner in the endocytic pathway \(^{184}\). Therefore it was observed that ectopic expression of the ubiquitin ligase c-Cbl, dramatically stimulates the sorting of endosomal EGFR to the lysosome, without affecting the rate of EGFR internalization \(^{185}\). Accordingly further studies in doa4 yeast mutant strains, defective in a deubiquitinating enzyme and therefore with low level of free ubiquitin, showed that endocytic cargo are missorted, suggesting that ubiquitin is involved in endosomal trafficking and sorting. This phenotype was shown to be reverted by ubiquitin overexpression \(^{186}\). Furthermore precursors of some vacuolar enzymes are monoubiquitinated. Inhibiting ubiquitination, by mutating cargo
ubiquitination sites or in enzymes of the ubiquitination machinery, blocks entry of the precursors into endosomal vesicles. Fusion of ubiquitin to the cytoplasmic domain of MVB vesicle cargo proteins that lack post-translational ubiquitination sites rescues the ability of the cargo to enter the vacuole lumen \(^{187}\).

These observations indicate that ubiquitin is necessary and sufficient to sort multiple cargo proteins into endosome \(^{165}\) and is required for degradation in the lysosome \(^{188}\).

### 1.2.3 Ubiquitin-receptors as endocytic adaptors

The ubiquitin signal on proteins in the biosynthetic-endocytic pathway is regulated to ubiquitin binding proteins (Ub-receptors). Ub-receptors proteins carry ubiquitin-binding domains, including UBA, UEV, UIM, CUE and GAT \(^{189,190}\). The ubiquitin binding domain of Ub-receptors are able to bind directly with weak affinity to a hydrophobic surface on ubiquitin composed of the residues Leu8, Ile 44 and Val70 \(^{177}\).

Ub-receptors are involved in internalization (e.g CIN85, c-Cbl), and are compartmentalized along the biosynthetic-endocytic pathway to regulate the trafficking of ubiquitinated cargo. The following are some examples of endocytic ub-receptors: UIM-containing proteins are involved in several aspect of endocytosis, such as in clathrin-coated vesicles binding (i.e. Eps15 and Epsin), or in internalization and sorting into the endosome (i.e. Vps27/Hrs, Hse1/STAM) \(^{191-194}\); as well UEV containing protein Vps23/TSG101 involved in vesicles fusion contains and viral budding \(^{162,195}\), or the c-Cbl UBA-containig protein is an ubiquitin ligase involved in ubiquitination of several immune receptors, RTK (i.e. EGFR) and other proteins \(^{184,196-201}\), the GTA containing protein Tom1 recruited with clathrin and endophilin to the endosomes.
1.3 The CUE Domain, an ubiquitin binding domain

The CUE domain family is one of the more recently described families of ubiquitin-binding domains, corresponding to a moderately-conserved domain of approximately 40-50 amino acids. In the sequence database there are roughly 50 eukaryotic proteins, such as yeast Cue1p, Vsp9; the mammals AUP1, AMFR and Tollip\(^{202}\) (Fig. 2.3a). The name of CUE family domain was based on the prototypical member in yeast, CUE1p protein (Coupling of Ubiquitin to ER degradation). The CUE1p was shown to be involved in recruitment of ubiquitin-conjugated Ubc6 and Ubc7 to an ER membrane complex involved in misfolded ubiquitinated proteins for exportation from the ER and proteasome degradation\(^{203}\).

The CUE domain was found in several yeast and mammalian proteins involved in ubiquitination process and endocytosis, such as in the C-terminal part of yeast Vsp9. The Vps9 is involved in the trafficking of ubiquitinated proteins trough the vacuolar/lysosomal system regulating the fusion of endosomal and Golgi derived vesicles. The Role of CUE-ubiquitin recognition was elucidated in the study of Vsp9 mutations\(^{204,205}\). Moreover the structure analysis of co-crystallized Vsp9 CUE domain with ubiquitin was elucidated revealing similarity to UBA domain (Ubiquitin Associated domain), as well the structure of the CUE-ubiquitin complex\(^{206,207}\).

Multiple alignment of CUE domains combined with structural features of CUE of Vsp9\(^{206}\) showed a three α-helical bundle with a conserved hydrophobic motif (MFP) and a second (LL), near the its C-terminus on the third α-helices and in the middle of the first α-helices (LL) (Fig. 2.3b). Tollip has both hydrophobic motifs on the surface of its CUE domain.

In summary, the CUE domain was shown to play a role in protein ubiquitination processes and in particular in regulation of endocytosis and trafficking of ubiquitinated cargo proteins in the endosome\(^{208,209}\).
Figure 2.3. CUE domain alignment and structure

a. Alignment of CUE domains from mammals and yeast proteins. b. Views of Vsp9 CUE domain structure, from the top (left) and from the side (right). The structure of Vsp9 CUE domain is composed of three α-helices forming a globular structure.
2. Material and methods

Cell culture
HeLa, HEK HEK 293T, HeLa, NIH3T3 and MEFs were cultured in DMEM Glutamax (Gibco) supplemented with 10% fetal calf serum. 293-Flag IL-1RI (G9) cells stably expressing the Flag tagged hIL-1RI were cultured in 2% FCS DMEM-NUT F-12 (Gibco). EL-4.6.10 cells were obtained from cultured in RPMI (Gibco) supplemented with 10% fetal calf serum, 50µM β-mercaptoethanol. All cells were cultured in presence of 100 µg/ml of penicillin/streptomycin mix (Gibco).

Reagents and antibodies
The source of the various antibodies used in this study is as follows: anti-Flag/M2 and anti-VSV (Sigma), anti-Xpress™ (Invitrogen), anti-ubiquitin antibodies P4D1 (SantaCruz) and anti-FK1 (Affiniti Research Products). Tollip antibody and serum against Tollip were made by rabbit immunization against GST-Tollip monoclonal anti-ubiquitin (P14D), anti-IRAK-1, were purchased by Santa Cruz. Express antibody was purchased by Invitrogen. Antibody anti-Flag M2 were purchased by SIGMA. Recombinant human IL-1β was purchased from Alexis (Lausanne) and and mouse recombinant IL-1β from SIGMA. GST-ubiquitin was obtained from BostonBiochem (Alexis)

Expression vectors
Expression vectors encoding Flag IL-1RI, Flag IL-1RAcP, IRAK-1 D340N (kinase dead), VSV Tollip and VSV Tollip deletion constructs with VSV Tag and in pCDN4/His max C (Invitrogen) have been previously described. Kinase dead IRAK-1 was used because wild type IRAK-1 is auto phosphorilated upon overexpression and interacts poorly with Tollip. The CUE mutant of Tollip (Tollip MF241-242AA) was generated by double PCR and inserted into a pCRIII vector (Invitrogen) with a N-terminal VSV tag. HA-ubiquitin and the ubiquitin mutant vectors (KO, K63, and K48) were kind gifts of D. Bohmann (Institute of Toxicology, Germany) and ZJ Chen (University of Texas Southwestern Medical Center, Texas), respectively. Flag and VSV-tagged Tom1 were cloned in pCR3 by PCR on EST clone (Invitrogen).
**Retroviral infections**

Tollip, or Tollip MF/AA was cloned into retroviral pMSCV vector. 10 cm plates of HEK 293T cells were cotransfected with Calcium phosphate methods with 10µg of pMSCV retroviral vector carrying the cDNA of GAG-POL with Tollip constructs, 1.5 µg pCG vector carrying a receptor for viral adherence and 10µg of the Hit60 vector encoding for viral capsid proteins. The cells were washed after 8 hours and stimulated with 10nM of Butyric Acid overnight. After washing cells were left for 24 hours before harvesting the viral supernatant. The viral supernatant were filtered and added with 1mM of polybren (Sigma) on cells of interest (HeLa, MEFs) and left overnight. After 24 hours the cells were selected and cultured in presence of 50µg/ml of puromycin (Sigma).

**Immunoprecipitation and Western Blot**

For overexpression HEK 293T (10 cm dish) cells were transfected with calcium phosphate method and lysed on ice with NP-40 1% Buffer, containing 250mM NaCl2, 20 mM Tris HCl pH 7.4, 1% NP-40 and complete protease inhibitor (Boehringer) or RIPA Buffer with 250mM NaCl, 20 mM Tris HCl pH 7.4, 0.1% SDS, 1% NP-40, 0.5% Deoxycholic acid and complete protease inhibitor. Lysates were precleared with 6B sepharose beads for 1 hour at 4°. For endogenous immunoprecipitation 100x10^6 EL-4.6.10, NIH3T3 and HeLa cells fibroblast were stimulated with 50ng/ml of mouse or human recombinant IL-1β. Cell lysates were first precleared with 6B sepharose beads, then incubated with 20μl of 50% slurry G Proteins Beads (Amhersam) for 4 hours at 4° with the following antibodies 1μg of M2 (Sigma), anti-VSV 2μg (Roche). For His pull down transfected HEK 293T cells (10 cm dish) were lysed by syringe in 6M Guanidine, 20mM Imidazole Buffer and load on nickel charged on 30μl of 50% slurry beads ProBond Resin (Invitrogen) for 2 hours at room temperature on rocking disk. For immunoblotting of the immunoprecipitates and whole-cell extracts were resolved on SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to Hybond–P membranes (Amersham). The membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase–conjugated antibodies to rabbit or mouse IgG using the Enhanced Chemiluminesence (ECL) Western blotting system (Amersham).
2-D gel electrophresys analysis

Immunoprecipitates performed as described before were eluted in 50 µl of 20 mM Tris pH8, 100mM EDTA, 0.5M NaCl resuspended in 150 µl of 9.0 M Urea, 4% (w/v) CHAPS (Sigma), 65 mM 1,4-dithio-DL-threitol, 0.8 % (v/v) Resolytes 4-8 (BDH, Poole, England) and 0.01% (w/v) bromophenol blue) that were used to rehydrate overnight 7-cm long IGP strips pH 4-7 (Amersham Pharmacia Biotech). Isoelectric focusing were performed on samples, resolved at a maximum voltage of 3500 V, until a Volt-hour count of 35'000 was reached. Second dimension were resolved by SDS-PAGE using 12% polyacrylamide gel done as described [Hochstrasser et al.]. Gel was analyzed by Silver Staining or by Western blotting.

Ubiquitin Binding Assays

To assay monoubiquitin binding, GST-ubiquitin (3µg) was immobilized on glutathione-Sepharose beads and then incubated with HEK 293Tcell extracts containing either VSV-Tollip or VSV-Tollip MF/AA in 1% NP-40 Lysis buffer for 2 hours at 4°C. The beads were washed 5 times in NP-40 Lysis buffer and bound proteins eluted with SDS-PAGE sample buffer.

Immunofluorescence Microscopy

Immortalized wild-type, Tollip-/-, or Tollip-/- MEFs stably expressing Tollip, Tollip MF/AA or empty vector were seeded on coverslips in 6 well dishes. The following day MEFs were transfected with IL-1RI (2µg) or IL-1RAcP (2µg) using TransIT-LT1 Transfection Reagent (Mirus Corporation). 24 hr later, cells were labeled at 4 °C or 37 °C with anti-IL-1RI antibody, 6B5, (Pharmingen) for 1hr, followed by stimulation with human IL-1β (50 ng/ml) at 37°C. Cells were then washed with PBS, fixed with 3.7% formaldehyde in PBS for 12 min, washed and permeabilized with PBS containing 2% goat serum and 0.1% saponin for 30 min (“antibody feeding” immunostaining technique). The cells were further incubated with AlexaFluor-488-conjugated antibodies against rat IgG (Molecular Probes). For costaining of internalized IL-1RI with LBPA, after IL-1RI labeling, cells were incubated with anti-LBPA (a kind gift of Jean Gruenberg, University of Geneva, Switzerland) followed by AlexaFluor 546 conjugated antibodies against mouse IgG (Molecular Probes). Tollip was labeled by incubation of wildtype or Tollip reconstituted MEFs with anti-Tollip antibodies (diluted 1:2000), followed by AlexaFluor-488-conjugated antibodies against rabbit IgG (Molecular Probes). Colocalization of Tollip was determined by costaining with one of the following
antibodies: Lamp1, Lamp2, LBPA or EEA1 antibodies. To label early endosomes MEFs were incubated with 20mM conjugated dextran for 10 min. The coverslips were mounted in FluorSave™ (Calbiochem) and images were taken using an inverted confocal laser-scanning microscope (LSM 510; Carl Zeiss).
3. Results and discussions

3.0 Objectives

Rational:
Tollip is composed of N-terminal region that binds Tom1, a protein potentially involved in endosomal trafficking; a C2 domain that binds the phosphatidylinositol-phosphates (PtdIns(3)P and PtdIns(3,4,5)P ); and a CUE domain that binds ubiquitin, and is necessary for the binding to IL-1RI. These features strongly suggested a role for Tollip as an endocytic adapter.

Overall objectives
Study the potential role of Tollip as endocytic adapter by focusing on the following aspects.

a. Characterization of Tollip CUE domain
   Ubiquitin binding domains are necessary to recognize ubiquitinated cargo and are also often involved in cis-ubiquitination of the protein.
   1) Confirm the ubiquitin binding capacity of the CUE domain of Tollip.
   2) Determine if Tollip is ubiquitinated, and if the CUE domain is required.
   3) Generate ubiquitin deficient mutant of the CUE domain.

b. Involvement of Tollip in IL-1R endocytosis.
   1) Determine if the IL-1RI is ubiquitinated.
   2) Determine if Tollip/IL-1RI interaction is ubiquitin-dependent.
   3) Study the localization of IL-1RI and Tollip.
   4) Evaluate the role of Tollip’s CUE domain in IL-1RI trafficking and degradation.
3.1 Tollip CUE domain binds ubiquitin species

The CUE domains from different organisms have conserved similarity in sequence and structure. Biochemical studies performed \textit{in vitro} with CUE domains from different proteins, including Tollip, showed that the CUE domain non-covalently binds monoubiquitin.

To confirm this, we carried out pull down experiments using His-ubiquitin (Fig. 2.4a) or GST-Ub (see Fig. 2.4b). HEK 293T cells were cotransfected with His-ubiquitin and VSV-Tollip or VSV-Tollip\textsubscript{ΔCUE}. Cell extracts were lysed in a 1% NP-40 lysis buffer, followed by His-Pull down using Ni\textsuperscript{2+} charged beads. His-ubiquitin associated proteins were analyzed by Western Blotting with anti-VSV antibody, showing that Tollip interacts with ubiquitin via its CUE domain (Fig. 2.4a).

To determine if the CUE domain of Tollip also binds to polyubiquitin chains we incubated GST and GST-Tollip with ubiquitin K48 or K63 linked polychains. The GST proteins were pulled down with Glutathion beads. Western Blot analysis with anti-ubiquitin antibody, revealed that Tollip binds both ubiquitin K48 and K63 linked polychains (Fig. 2.4b).

These findings indicated that Tollip is able to bind, at least in vitro, both monoubiquitin and polyubiquitin forms.

\textit{Figure 2.4. The CUE domain of Tollip bind non-covalently monoubiquitin and ubiquitin polychains.}
a. HEK 293T cells were transfected His Xpress\textsuperscript{TM} ubiquitin and with VSV-Tollip or VSV-Tollip\textsubscript{ΔCUE}. Cell extract were incubated with Nickel charged Beads in 1% NP-40 Lysis Buffer over night at 4\degree. Cell extracts and His Pull down samples were analyzed with anti-VSV antibody. b. Immobilized recombinant GST-Tollip or GST on glutathione-beads was used pull down ubiquitin K48 and K63 linked polychains. The pull down assay was analyzed by Western Blot with anti-ubiquitin antibody.
3.2 Tollip is ubiquitinated

Proteins containing an ubiquitin-binding domain are themselves often ubiquitinated. This due to the fact that binding of ubiquitin and ubiquitinated proteins allows the formation of complex via ubiquitin with ubiquitinating enzymes that catalyze their ubiquitination. Therefore is possible that Tollip could also be ubiquitinated.

Tollip has a predicted molecular weight of 28 kDa, however in Western Blotting we often detected an upper species (8-10 kDa higher), that cross-reacted with anti-Tollip antibodies. This band was more evident, together with others minor upper species, upon overexpression of Tollip (Fig. 2.5a). However, this band was also observed at the endogenous level in extracts from EL-4 cells (data not shown) or from various mouse tissues (Fig. 2.5b).

The size of the modification (8-10 kDa) and the pattern of the modifications suggested that Tollip might be conjugated with ubiquitin or by a ubiquitin-like moiety (i.e. SUMO, NEDD8).

To address this possibility we carried out several immunoprecipitation experiments. We transfected VSV-Tollip in to HEK293T cells in the presence or absence of HA-ubiquitin. Cell lysates were prepared using RIPA buffer. These relatively harsh conditions using, were used to minimize non-covalent binding of ubiquitin or other binding partners, then used to perform immunoprecipitation with anti-VSV antibody.

Western Blot analysis with anti-ubiquitin or anti-HA antibody of VSV immunoprecipitates, revealed the same typical smear-like pattern of ubiquitinated proteins. The modification of Tollip at 8-10 kDa higher (37-40 kDa) (detected with the anti-VSV and anti-Tollip antibodies) was not recognized by the anti-ubiquitin antibody, however was detected by anti-HA antibody, suggesting that this band is likely monoubiquitin. That it was not detected by anti-ubiquitin antibody may be due to reduced accessibility of the mono-ubiquitin epitope for the antibody. No signal was detected using antibodies against SUMO-1 and SUMO-2 (Data not shown), supporting that Tollip is ubiquitinated.

We also performed 2D gel analysis of Tollip. HEK 293T transfected with His-Tollip, were lysed under denaturing condition (6M guanidium), thereby excluding all non-covalent interactions with ubiquitin. The His-Tollip pull down was resolved by 2D-SDS PAGE and analyzed by Western Blotting with anti-Tollip or anti-ubiquitin antibodies (Fig. 2.6a). Multiple spots were crossreactive with anti-Tollip antibody, including a spot at the predicted size of His-Tollip (30-32 kDa) (Fig.2.6b, arrows 1), as well several spots with increasing size and isoelectric point (Fig. 2.6b, arrows2). These spots correspond to the pattern expected for increased size and isoelectric pattern due to polyubiquitin chains (Fig. 2b, arrows 2). In fact,
these spots cross-reacted with both anti-Tollip and ubiquitin antibody. Interestingly, the spots recognized by Tollip antibody at 30-32 kDa have different isoelectric points, suggesting that Tollip may also be modified by phosphorylation (fig. 2.6b, arrows1). Similar results were observed with immunoprecipitated VSV-Tollip cotransfected with HA-ubiquitin in 293T cells (Fig. 2.6c).

Together these results indicated that Tollip is modified by a polyubiquitin chain already at the endogenous level, and that overexpression enhance its ubiquitination.
Figure 2.5. Tollip is modified
a. Cell extracts of HEK 293T cells transfected with VSV-Tollip were analyzed by Western Blot with a Rabbit polyclonal anti-Tollip antibody. b. Extracts of indicated murine tissues were analyzed by western Blot with anti-Tollip antibody.

Figure 2.6 Tollip is ubiquitinated
VSV-Tollip was transfected with HA-Ubiquitin in HEK 293Tcells. Cell extracts were prepared in RIPA buffer, and immunoprecipitated with an anti-VSV antibody at 4° overnight. The immunoprecipitations were resolved by 1 dimensional (a) and 2D-SDS PAGE (c). b. His-tollip was transfected in HEK 293Tcells and lysed in Guanidine 6M Buffer. Cell extracts were used to perform Pull down with Ni²⁺ charged beads for 2 hours at room temperatures. His-pull down were analyzed by Western Blot with anti-HA, anti-ubiquitin or anti-Tollip antibodies.
To determine if Tollip ubiquitination is induced in a ligand dependent manner, Tollip was immunoprecipitated from HeLa cells stably transfected with VSV-Tollip (Fig. 2.7a) or wild type mouse NIH3T3 fibroblasts (Fig. 2.7b) before and after IL-1β. These experiments showed that Tollip is ubiquitinated, but not in an IL-1β independent manner. Therefore, Tollip differs from other ubiquitinated proteins in the IL-1β signaling cascade, (i.e. TRAF6 and IRAK-1) which are ubiquitinated in a stimulus dependent manner.

**Figure 2.7 Tollip is constitutively ubiquitinated.**

a. Stably retroviral transfected VSV-Tollip HeLa cells were stimulated with 50ng/ml human IL-1β at indicated time points. Cell extracts were lysed in RIPA lysis buffer and immunoprecipitated overnight with VSV antibody. Cell extracts were analyzed by Western Blot with antibodies as indicated. b. Wt NIH3T3 fibroblasts were stimulated with 20ng/ml with mouse IL-1β at indicated time points. Cell extracts were prepared in RIPA Buffer and immunoprecipitated with anti-Tollip antibody at 4° overnight. Immunoprecipitations and cell extracts were analyzed by Western Blot with anti-Tollip and anti-ubiquitin antibodies.
3.3 Tollip CUE in necessary for Tollip ubiquitination

To determine the requirement of Tollip’s CUE domain for its ubiquitination, VSV-Tollip deletion mutants (Fig. 2.8, right panel) were transfected in HEK 293T cells. Transfected cells were lysed with RIPA Buffer and VSV- Tollip constructs were immunoprecipitated using an anti-VSV antibody (Fig. 2.8). Western Blot analysis of the VSV immunoprecipitations revealed that the Tollip mutant lacking the complete C-terminal region and the CUE deletion domain were not ubiquitinated. Interestingly, the C2 deletion mutant showed a reduced ubiquitination.

These results confirm that the CUE domain is necessary for Tollip’s ubiquitination. The reduced ubiquitination of the C2 deletion mutant could be explained by the fact that, the C2 region is rich in lysine residues that could be potential sites of ubiquitination.

To address which lysine or lysines are conjugated by ubiquitin and the topology of the attached chains we initiated MS-MS sequencing experiments. To obtain sufficient amounts of pure Tollip protein, HEK 293T cells were transfected with VSV-Tollip. Immunoprecipitated VSV-Tollip was resolved by SDS-PAGE and stained with coomassie brilliant blue. This showed a relatively pure band of Tollip and a faint band of potentially modified Tollip (37-40 kDa), as well upper bands of higher molecular weight. Several of these bands were excised out and digested with trypsin for MS-MS sequencing. Unfortunately, we could only detect peptides from Tollip and ubiquitin peptides, but no Tollip peptide with covalently bound ubiquitin, and therefore could not determine the site of ubiquitination. However, the upper bands corresponded to peptides from K48 ubiquitin polychains. It is unlikely, that Tollip is conjugated to K48 ubiquitin polychains because the Tollip’s stability. It is perhaps more probable that these K48 polychains were not conjugated to Tollip, but were derived from residual non-covalent binding to the CUE domain as result of the purification.
Figure 2.8. Tollip is ubiquitinated via its CUE Domain.
a. VSV-Tagged Tollip constructs (Wt, ΔNT, ΔCUE, ΔC2, ΔCT) were transfected HEK 293T cells in. Cell extract were prepared in RIPA buffer, and used for immunoprecipitation with an anti-VSV antibody. Immunoprecipitations and cell extracts were analyzed by Western Blot with anti-ubiquitin and VSV antibody.
3.4 Generation of ubiquitin deficient mutant CUE

In order to elucidate the function of the CUE domain we generated a CUE domain mutant that is no longer able to bind ubiquitin. Based on structure analysis of the yeast Vsp9 CUE domain and multiple alignments of CUE domains, a conserved motif was observed. This CUE conserved motif is called MFP (a.a 241-243) and is present between \( \alpha \)-helix1 and \( \alpha \)-helix2, at the contact surface between ubiquitin and the CUE. We mutated both methionine 241 and phenylalanine 242 of MFP motif in Tollip CUE domain to alanines (Fig. 2.9a). To test the ubiquitin binding capacity of this Tollip mutant, called TollipMF/AA, we performed \textit{in vitro} GST-ubiquitin Pull down assays. Cell extracts prepared from 293T cells transfected with VSV-tagged Tollip or TollipMF/AA (Fig. 2.9b), were incubated with immobilized GST or GST-ubiquitin on glutathione beads. The GST or GST-ubiquitin associated proteins were analyzed by Western Blotting with anti-VSV antibody, showing that TollipMF/AA mutant cannot bind ubiquitin. The Tollip MF/AA mutation impaired ubiquitination of Tollip (Fig. 2.9c.).

These results suggested that ubiquitin binding capacity of Tollip’s CUE is necessary for self-ubiquitination, supporting the hypothesis that the CUE domain can recruit ubiquitinated proteins and ubiquitin machinery required for its ubiquitination. Therefore we generated an useful tool to study and characterize the function of Tollip’s CUE and ubiquitin binding capacity.
Figures 2.9 TollipMF/AA loses the ubiquitin binding capacity and ubiquitin modification

a. Structural model of the CUE-Ubiquitin complex, showing the position of M241 and F242 mutated residues at the hydrophobic surface between the CUE domain and Ubiquitin (Kang et al., 2003). 

b. GST-Ubiquitin Pull down assay. Immobilized GST or GST-ubiquitin were incubated 2 hours with VSV-Tollip constructs obtained from HEK 293T cells transfected with VSV-Tollip wt and the VSV-Tollip MF/AA mutant lysed in NP-40 1% Buffer. The GST Pull Down was analyzed by Western analysis with anti-VSV and anti-GST antibodies.

c. VSV-Tollip wt or the VSV-Tollip MF/AA mutant were transfected with HA-Ubiquitin in HEK 293T cells. Cell extracts were prepared in RIPA buffer, and immunoprecipitated with an anti-VSV antibody at 4° overnight. The immunoprecipitations were analyzed by Western analysis with indicated antibodies.
3.5 Tollip’s CUE-Ubiquitin dependent IL-1R interaction

Previous studies have already shown that Tollip forms a complex with IL-1R chains following IL-1β stimulation and this interaction is dependent on the CUE domain. I confirmed these results by cotransfecting Flag-IL-1R and IL-RAcP (IL-1Rs) with VSV-tagged Tollip deletion mutants (Fig. 2.10a, right panel). The Flag immunoprecipitates were analyzed by Western Blot analysis with anti-VSV antibody (Fig. 2.10a). Interestingly, we observed that TollipMF/AA mutant only weakly interacted with IL-1Rs, as shown in Figure 2.10b (Fig. 2.10b).

Tollip was also shown to bind to IRAK-1 via its C-terminal CUE domain. To determine if the TollipMF/AA impaired IRAK-1 binding, we performed immunoprecipitation of VSV tagged Tollip or TollipMF/AA and IRAK-1, showing similar binding of IRAK-1 to Tollip or TollipMF/AA (Fig. 2.10c).

Together these results indicate that Tollip’s ubiquitin binding capacity is required for a high affinity interaction between IL-Rs and Tollip, but not for IRAK-1 interaction.
Figure 2.10 Tollip CUE domain and its ubiquitin binding are crucial for IL-1R binding.

a. VSV-Tollip deletion mutants (right panel) were cotransfected with Flag IL-1RI and IL-1RACp in HEK 293T cells. Cell extracts were immunoprecipitated with anti-Flag antibody. Cell extracts and immunoprecipitates were analyzed with anti-VSV and anti-Flag antibodies. b. Tollip and Tollip MF/AA mutant were coimmunoprecipitated with Flag IL-1R using Flag antibody. c. IRAK-1 was co-immunoprecipitated with both Tollip and Tollip MF/AA mutant analyzed by Western Blot with anti-VSV and anti-IRAK-1 antibodies.
3.5 IL-1R chains ubiquitination

As the ubiquitin-binding capacity of Tollip appeared to be important for high affinity binding to the IL-1RI, we wanted to determine if IL-1R is ubiquitinated and if this occurs in ligand-dependent manner.

To address this question IL-1RI was immunoprecipitated from G9-293 cells, a cell line stably transfected with Flag tagged IL-1RI, before or after stimulation with IL-1β at different time points (to 4 hours). Cell lysates were prepared in relative harsh conditions (RIPA Buffer) and Flag-IL-1RI was immunoprecipitated using anti-Flag antibody. Western Blot analysis of the Flag immunoprecipitation with anti-ubiquitin antibody revealed that the IL-1RI was rapidly ubiquitinated following IL-1β stimulation and remained so, although with decreasing intensity, over a time course of 4 hours (Fig. 2.11a).

To determine if the coreceptor IL-1RacP is also ubiquitinated, we cotransfected HEK 293T cells with HA-ubiquitin, IL-1RI and/or IL-1RacP. This experiment showed that both IL-1RI and IL-1RacP were ubiquitinated, which was enhanced by coexpression of HA-ubiquitin (Fig. 2.11b). Interestingly, IL-1RacP was ubiquitinated to a significantly greater extent than the IL-1RI chain alone (Fig. 2.11b).

To determine the topology of the ubiquitin conjugated to IL-1Rs, we used several HA-tagged ubiquitin mutants (Fig. 8c, lower panel): a lysine free ubiquitin unable to form poly-ubiquitin chains (UbKo mutant), or ubiquitin mutants (UbK48, UbK63) which are lysine free except at the indicated positions allowing the formation of only K48 or K63 polychains respectively. These ubiquitin mutants were coexpressed with the Flag IL-1Rs in HEK 293T cells (Fig. 2.11c). The Flag immunoprecipitates were analyzed by Western Blot with anti-HA-antibody. This experiment revealed the same ladder-like ubiquitin pattern was detected with each of the HA-tagged ubiquitin mutants, including the UbKo mutant. These findings suggested that IL-1R chains were conjugated by multiple monomeric ubquitins rather than a polychain (K48 or K63) (Fig. 2.11c). Furthermore, immunoprecipitated ubiquitinated Flag IL-1Rs, from transfected HEK 293T cells (Fig. 8d, left panel) or from IL-1β stimulated Flag IL-1RI G9-293 cells (Fig. 2.11d, right panel) were shown to be recognized with the P4D1 monoclonal anti-ubiquitin antibody, which recognizes monoubiquitin as efficiently as polyubiquitin, but not with the anti-ubiquitin antibody FK1 clone specific for polyubiquitin. As a control, overexpressed Flag-TRAF6, shown to be conjugated by K63-linked ubiquitin, was detected with both antibodies (Fig. 2.11d, left panel).

In summary, these results suggest that IL-1RI is monoubiquitinated in a ligand-induced
manner, as known for other endocytosed receptors (i.e. EGFR)

Figure 2.11 The IL-1R chains are monoubiquitinated

a. HEK293-Flag IL-1RI (G9) cells were stimulated or not with 50ng/ml of IL-1β and lysed in RIPA Buffer. The cell extracts were immunoprecipitated with anti-IL-1RI antibody. Cell extracts were analyzed with an anti-ubiquitin antibody (PD41). b. Flag IL-1R and IL-1RacP were expressed together or alone in presence of HA-ubiquitin in HEK 293T cells. Cells were lysed in RIPA buffer and immunoprecipitated with anti-Flag antibody. Cell extracts and immunoprecipitations were analyzed with anti-ubiquitin and anti-Flag antibodies. c. Flag-IL-1R chains were cotransfected with ubiquitin mutants lacking lysine residues, with only K48 or K63 linked polychains (lower panel). Lysates were lysed in RIPA and immunoprecipitated with anti-Flag antibody. The immunoprecipitation were analyzed with anti-ubiquitin and anti-Flag antibodies. d. HEK 293 were transfected with Flag tagged TRAF6 or IL-1R chains. The cell extracts were immunoprecipitated with anti-Flag antibody. Immunoprecipitates were analyzed with different anti-ubiquitin antibodies, PD41 and FK1. e. IL-1R were immunoprecipitated HEK293-Flag IL-1RI (G9) cells as described above. Immunoprecipitations were analyzed with different anti-ubiquitin antibodies clones PD41 and FK1.
3.4 Tollip and IL-1R internalization

It has been known for some time that IL-1β stimulation of IL-1RI triggers its internalization. However the underlying mechanisms driving its intracellular trafficking and degradation are unknown.

To begin to elucidate the mechanism of IL-1β dependent internalization of IL-1RI and the role of Tollip in this process, we developed an immunofluorescent confocal microscopy assay using wild type or Tollip deficient MEFs. Wild type MEFs were transiently transfected with low levels of Flag IL-1RI and IL-1RAcP, as IL-1RI was not detectable endogenously with available antibodies. Transfected cells were immunostained by “antibody feeding” with a specific IL-1RI antibody (see material and methods section), followed by confocal microscopy analysis. The images of the unstimulated MEFs showed plasma membrane localization of IL-1RI (Fig. 2.12a), whereas after stimulation for 30 or 60 minutes with IL-1β, IL-1RI was observed an intracellular (Fig. 2.12b).

IL-1RI and Tollip were shown to interact rapidly and transiently, suggesting that Tollip is part of the membrane proximal signaling complex. To determine if Tollip also colocalizes with internalized IL-1RI, wild type MEFs were costained for internalized IL-1RI and Tollip, as described above. The confocal images showed colocalization of IL-1RI and Tollip in intracellular structure after 30 minutes of IL-1β stimulation (Fig. 2.12b), suggesting that Tollip is localized to endosomes like IL-1RI.
Figure 2.12 IL-1R is rapidly internalized after IL-1 stimulation and colocalizes with Tollip.

a. Mouse embryonic fibroblasts (MEFs), transfected with Myrus lipofectamine Flag-IL-1RI and IL-1RacP, were incubated with antibodies against IL-1RI for 1 hour at 4 °C and then incubated at 37 °C for 1hr in medium stimulated with 20ng/ml IL-1β (30 and 60 min). Central sections of the cells imaged by confocal microscopy are shown. b. The MEFs were transfected, stimulated and stained with antibodies against IL-1RI and Tollip as indicated above. After permeabilization, the cells were stained with a rat 488-conjugated (green) and rabbit Cy5 secondary antibody (blue).
To confirm that Tollip is localized to endosomes, Tollip was colocalized with different endosomal markers.

As tool to facilitate colocalization, immortalized Tollip\(^{-}\)/ MEFs by were retrovirally reconstituted with empty pMSCV vector or pMSCV VSV-Tollip. The levels of Tollip in these cells are comparable to endogenous level. Wild type or reconstituted MEFs were costained for Tollip using anti-VSV or anti-Tollip antibodies together with endosomal specific markers (Fig. 2.13). The diffuse and punctuate staining pattern of Tollip is specific as evidenced by absence of Tollip staining in Tollip deficient cells (data not shown). No or very little colocalization was detected with early endosome antigen 1 (EEA1) (Fig. 2.13 a-c) or with fluorescent dextran after a 10 min pulse to label early endosome populations (Fig. 2.13 d-f). Also, no colocalization was detected with the trans Golgi marker, Golgin 97 (Fig. 2.13 g-i). Partial colocalization was detected with the phospholipid, lysobisphosphatidic acid (LBPA), which is abundantly localized to the luminal membranes of late endosomes/MVBs (Fig. 2.13 j-l). In contrast to the above stainings, Tollip significantly colocalized with the late endosome/lysosome markers, Lamp1 and Lamp2 (Fig. 2.13 m-r). The diffuse staining of Tollip, that does not colocalize with Lamp1 or Lamp2 is likely to be cytoplasmic, as suggested by fractionation experiments in which Tollip partitioned equally between membrane and cytosolic fractions (Kim Burns, personal communication). These results showed that Tollip is also localized in late endosome.
Figure 2.13. Tollip localizes to late endosomal compartments.
Mouse embryonic fibroblasts (MEFs) wild type and retrovirally transduced with pMSCV VSV-Tollip were permabilized and costained with antibodies against VSV (panel a) or Tollip (panels d,g,j,m,p) and EEA1 (panel b), Dextran pulse (panel e), Golgin97 (panel h), LBPA (panel n), LAMP1 and LAMP2 (panel n,q). All were stained with Alexa-conjugated secondary antibody 488 (green), 543 (red). Nuclei were stained with Draq5.
To determine the role of Tollip in trafficking of IL-1RI, we monitored by confocal microscopy endosomal localization of IL-1Rs after IL-1β stimulation in wild type and Tollip<sup>−/−</sup> MEFs.

Both Wild type and Tollip<sup>−/−</sup> MEFs were transiently transfected with low levels of IL-1RI and IL-1RAcP. Transfected cells were costained for IL-1RI with and EEA1 or LBPA (Fig. 2.14 and data not shown). Then we analyzed the IL-1RI trafficking induced by IL-1β stimulation in these cells (Fig. 2.14 a).

Staining of transfected wild type and Tollip<sup>−/−</sup> MEFs was done after 1 hour or 3 of IL-1β stimulation, showing colocalization of IL-1RI with the late endosomal marker, LBPA. In contrast, no staining of the IL-1RI with early endosome marker EEA1 was observed (Fig.2.14a). However, after 1 hour of IL-1β stimulation, we observed few colocalization of IL-1RI with LBPA in wild type MEFs and slightly more in Tollip<sup>−/−</sup> cells (Fig. 2.14a left panels), suggesting that the IL-1RI may be accumulating in late endosome and not efficiently sorted to the lysosome. Therefore to investigate the consequences of accumulating internalized IL-1RI in Tollip<sup>−/−</sup> MEFs, we examined the fate of internalized IL-1RI over time. Strikingly 3 hours after IL-1β stimulation, when degradation of the IL-1RI was essentially complete in wild type cells, a substantial amount of IL-1RI could still be detected in Tollip<sup>−/−</sup> MEFs, the majority of which colocalized with enlarged LBPA positive endosomes (Fig. 3c right panels). As a control we used fluorescent dextran as a soluble marker showing no general defect in bulk transport from early to late endosomes in wild type and Tollip<sup>−/−</sup> MEFs (Fig. 2.14b). The cells were also used for control experiment by pulsing the cells with rat IgGs as fluid phase marker, showing that endocytosed IgGs were transported to lysosomes and degraded similarly in wild type and Tollip<sup>−/−</sup> MEFs (data not shown), suggesting that Tollip deficiency does not cause a general defect in degradation. Taken together, the experiments described so far indicate that Tollip is required for efficient lysosomal sorting from late endosome, and thereby for degradation of the IL-1RI.
Figure 2.14 Internalized IL-1RI is accumulated in the late endosome in Tollip\textsuperscript{−/−} MEFs, but not in wild type cells.
a. Wt an Tollip\textsuperscript{−/−} mouse embryonic fibroblasts (MEFs) were transfected with Myrus lipofectamine Flag-IL-1RI and IL-1RacP. After 24 hours the cells were incubated with antibodies against IL-1RI for 1 hour at 4 °C and then incubated at 37 °C for 1 hr in medium stimulated with 20ng/ml IL-1β (1h and 3h). The cells were permeabilized and stained with an Alexa-488-conjugated secondary antibody (green). Central sections of the cells imaged by confocal microscopy are shown. b. MEFs were pulsed with fluorescent dextran (red). The cells were permeabilized and stained with antibody against LAMP1 and LBPA, and revealed with Alexa-488 (green) or 543 (red) conjugated secondary antibody. Nuclei were stained with Draq5.
To determine if Tollip’s CUE domain and its ability to bind ubiquitin have a direct role in IL-1RI trafficking and lysosomal sorting, we examined IL-1RI trafficking in MEFs expressing the TollipMF/AA mutant.

To address this question Tollip−/− MEFs were reconstituted with empty pMSCV, VSV-Tollip or VSV-TollipMF/AA and were costained for IL-1RI and LBPA, followed by stimulation for 3 hours with IL-1β. These cells were analyzed by confocal microscopy showing that, in TollipMF/AA mutant MEFs IL-1RI accumulated in enlarged LBPA positive structures (late endosome), similarly to the Tollip−/− empty vector reconstituted MEFs (Fig. 2.15). In contrast in Tollip reconstituted MEFs we do not observed this enlarged late endosomal structures with IL-1RI and LBPA staining.

These results indicate that Tollip’s CUE ubiquitin binding capacity plays a crucial role in IL-1RI trafficking to the lysosome, as shown for other endocytic adapters containing ubiquitin-binding domains.

Figure 2.15 Tollip deficiency or MF/AA mutation leads to IL-1R increased accumulation in the late endosome
Mouse embryonic fibroblasts (MEFs) retrovirally transfected with pMSCV, pMSCV VSV-Tollip or VSV-TollipMF/AA were transfected using Myrus lipofectamine with Flag-IL-1RI and Flag-IL-1RacP. After 24 hours the cells were incubated with antibodies against IL-1RI for 1 hour at 4 °C and then incubated at 37 °C in medium containing 20ng/ml of IL-1β for 3 hours. After permeabilization cells were stained with an Alexa-488-conjugated secondary antibody (green) and LBPA (red). Central sections imaged by confocal microscopy are shown.
3.5 Tollip and IL-1Rs binding proteins for signaling and endocytosis

To further investigate the mechanisms by which Tollip may control endocytosis of IL-1Rs, we initiated studies to identify and characterize other Tollip binding proteins (i.e. Tom1).

3.5.1 Tom1 as endocytic adaptor interacting with Tollip

Tom1 (Target Of Myb) was first described to be upregulated by the oncogene c-Myb. Tom1 is a member of a subfamily of protein that includes Tom1-like1 and 2 (Tom1-L1 and 2)\(^{215}\). These proteins are characterized by the presence of a VHS (Vps/Hrs/STAM) domain, ubiquitin-binding GAT domain (GGA and Tom1)\(^{190}\) and three motifs responsible for Clathrin Binding (CB) (Fig. 2.16). Tom1 and Tom1L1 molecules have structural and functional similarity with the GGA (Golgi-localizing, γ–adaptin domain homology, ARF-binding protein) proteins, a family of endocytic adapters necessary for the delivery of both biosynthetic and endosomal cargo to the lysosome \(^{216}\). Tom1-L1 was shown to bind to the Hrs (Hepatocyte growth factor-regulated tyrosine kinase substrate) and TSG101 (Tumor Susceptibility Gene 101)\(^{217}\), a component of the ESCRTI complex crucial for sorting and regulation of MVB formation. All these observations suggest a role for Tom1 and Tom1-L1 in endocytic trafficking.

Recently, Tollip and ubiquitin were identified as Tom1-interacting proteins in a yeast two-Hybrid screen, using the C-terminus of Tom1 (VHS and GTA) as bait. The interaction between Tom1 and Tollip was mapped to the N-terminus of Tollip \(^{218}\). We confirmed this interaction by cotransfecting HEK293T cells with Tom1 and Tollip full length or TollipΔNT (Fig. 2.17a). Tom1 interacts via its GAT domain (residues 212-312) with ubiquitin and Tollip, and ubiquitin competes with Tollip for this site \(^{218}\). Endophin, another Tom1 binding protein, induces early endosomal localization of Tom1 which is found in the cytosol in the absence of endofin Tollip overexpression \(^{219}\). It was also showed that Tom1 and Tollip are localized in early endosomal compartments with ubiquitinated proteins \(^{220,221}\).

These findings, together with our previous observation that Tollip is required for IL-1RI trafficking, strongly support the hypothesis that Tom1 and Tollip could act synergically in IL-1RI internalization and trafficking.
3.5.2 Tom1 binds IL-1Rs with Tollip

To access binding of Tom1 with IL-1RI, we carried out immunoprecipitation experiments. We cotransfected HEK 293T cells with Flag-IL-1Rs, VSV-Tom1 and/or VSV-Tollip. The cell extracts were used to perform immunoprecipitation with anti-Flag antibody. Flag immunoprecipitates were analyzed by Western blotting with anti-VSV and anti-Flag antibodies, showing that Tom1 is recruited to IL-1Rs, and that this interaction is slightly enhanced by the presence of Tollip (Fig. 2.17b). These results suggested that Tom1 is recruited to IL-1R with Tollip, and perhaps binds the receptor via by Tollip.

To map the Tom1 region that interacts with Flag-IL-1Rs, we generated different VSV tagged Tom1 deletion mutants. HEK 293T cells were cotransfected with Flag-IL-1Rs with togheter with one of VSV-Tom1 deletion mutants. Western Blot analysis of the Flag immunoprecipitates showed that the Tom1 GAT domain deletion mutant (Δ212-312) and the C-terminal deletion (Δ312-492) are required for maximal interaction with IL-1Rs, whereas deletion mutant of the VHS domain appeared to have no effect on binding IL-1Rs (Fig. 2.17c).

Interestingly, we observed that co-expression of Tom1 with Flag-IL-1Rs resulted in decreased expression levels of IL-1Rs as revealed by western Blot analysis. This apparent disappearance of IL-1Rs was enhanced by cotransfection with increasing amounts of Tom1, but blocked partially by addition of Tollip (Fig. 2.18a). Coexpression of Tollip or other signaling proteins (i.e. TRAF6, IRAK-1, TAK1) with IL-1Rs does not affect IL-1R levels like Tom1 (data not shown). To confirm that Tom1 specifically reduced IL-1R levels, we cotransfected Tom1 with TNF-RI, the levels of which remained unchanged (Fig. 2.18b). These preliminary results with Tom1, supported by previous description of Tom1 as an endocytic adapter, raised the hypothesis that Tom1 overexpression could drive IL-1Rs degradation. However to confirm this hypothesis, further experiments with proteases inhibitors, to block this effect of Tom1, will be required (experiments in progress).
Figure 2.17 TOM1 binds Tollip and the IL-1R complex

a. HEK 293T cells were cotransfected with Flag-Tom1 and VSV-Tollip or TollipΔNT. Cell extracts were used for immunoprecipitation against Flag (left panel) or VSV (right panel) proteins. b. HEK 293T cells were cotransfected with Flag-IL-1R chains and VSV-Tom1 and VSV-Tollip. Cell extract were used for immunoprecipitation against Flag proteins with anti-Flag antibody. c. VSV tagged deletion mutants of Tom1 were cotransfected with Flag-IL-1R chains in HEK 293T cells. Cell extracts were used for immunoprecipitation against Flag proteins with anti-Flag antibody. Both cell extracts and immunoprecipitations were analyzed by Western Blot with anti-Flag and anti-VSV antibodies.
Figure 2.18 Tom1 overexpression reduces the IL-1Rs expression level.
a. HEK 293T cells were cotransfected with Flag-IL-1Rs and VSV-Tollip and increasing amounts of Tom1. Cell extract were prepared in RIPA Buffer and used for immunoprecipitation with anti-Flag antibody. Both cell extracts and immunoprecipitates were analyzed by Western Blot with the indicated antibodies. b. HEK 293T cells were cotransfected with Flag-IL-1R chains or VSV-TNF-R1 and VSV-Tom1 cell extracts were analyzed by Western Blot analysis.
3.5.3 Tollip interacts with TRAF6, and forms a complex with Rab proteins:

3.5.3.1 Tollip interact with TRAF6 in IL-1β dependent manner

TRAF6 is recruited to IL-1R membrane proximal signaling complex upon IL-1β stimulation, via interaction with IRAK-1. TRAF6 is a crucial platform protein for the activation of several pathways. Activated TRAF6 forms a complex with the E2 ligase (Ubc13/Uev1a) and via its RING domain, induces self ubiquitination of a by K63-linked polychain.

As TRAF6 is member of the IL-1R signaling complex and is also ubiquitinated, we wanted to investigate if Tollip interacts with TRAF6 (Fig 2.19a). We cotransfected Flag-tagged TRAF constructs (from TRAF 1 to 6) with VSV- Tollip in HEK 293T cells. Cell extracts were prepared and immunoprecipitated with anti-Flag antibody, followed by Western Blot analysis. This experiment showed that Tollip interacts with Flag-TRAF6, and to a lesser extent with Flag-TRAF2 and TRAF5.

To determine if Tollip and TRAF6 interacted in an IL-1β dependent manner, we immunoprecipitated endogenous TRAF6, before an after IL-1β stimulation at the indicated time points. Western Blot analysis showed that TRAF6 and Tollip interact rapidly and transiently after IL-1β stimulation (Fig 2.19b).

Figure 2.19 Tollip interact with TRAF6 in IL-1β dependent manner

a. HEK 293T cells were cotransfected with Flag Tagged TRAF1 to 6 and VSV-Tollip. Cell extracts were prepared and immunoprecipitated with anti-Flag antibody, followed by Western Blot analysis with anti-VSV and anti-Flag antibodies. b. EL-4 cells (10^8 cells X sample) were treated at indicated time points with IL-1β (50ng/ml) or left untreated. Cell extract were prepared in Hepes NP-40 lysis Buffer (0.2%) and immunoprecipitates with anti-Tollip antibody at 4° overnight. The immunoprecipitation were analyzed by Western Blot analysis with indicated antibodies.
3.5.3.2 Tollip modulates TRAF6 ubiquitination

To access interaction between Tollip and TRAF6 is mediated via Tollip’s CUE domain, Flag TRAF6 was coexpressed with VSV-Tollip deletion mutants in HEK 293T. The Flag immunoprecipitates revealed that Tollip lacking the C-terminal part or the CUE domain interacted less well with TRAF6 than Tollip or Tollip ΔNT (Fig. 2.22a), suggesting a role of the Tollip’s CUE domain in TRAF6 binding. Interestingly, we also observed an enhancement of TRAF6 ubiquitination modifications when coexpressed with Tollip, but not TollipΔCUE or VSV-TollipMF/AA (Fig. 2.20b). This suggested that Tollip via its CUE domain could induce, stabilize or protect from deubiquitination TRAF6 ubiquitination.

To further investigate if Tollip is required for IL-1β induced TRAF6 ubiquitination, endogenous TRAF6 was immunoprecipitated from Tollip+/− MEFs reconstituted with empty vector, VSV-Tollip or VSV-TollipΔCUE following stimulation with IL-1β. Western Blot analysis with anti-ubiquitin antibody revealed normal TRAF6 ubiquitination in Tollip deficient cells, as well as Tollip and TollipΔCUE mutants expressing cells (Fig. 2.20c), suggesting that Tollip is not essential for TRAF6 ubiquitination. However, we cannot exclude that Tollip and its CUE domains could modulate TRAF6 ubiquitination, to regulate the signal.

In facts, others proteins were shown responsible for TRAF6 ubiquitination, such as TAB2 and TAB3 125-126.

Figure 2.20 Tollip induces but is not essential for TRAF6 ubiquitination.

a. and b. HEK 293T cells were cotransfected with Flag Tagged TRAF6 (0.5µg per plates) and VSV-Tollip, TollipMF/AA, or TollipΔCUE. Cell extracts were prepared and immunoprecipitated with anti-Flag antibody, followed by Western Blot analysis with anti-ubiquitin and Flag antibodies. c. Tollip deficient MEFs retrovirally reconstituted with pMSCV VSV-Tollip or TollipΔCUE (20 cm dish X sample) were treated at indicated time points with IL-1β (50ng/ml) or left untreated. Cell extract were prepared in RIPA lysis Buffer and immunoprecipitated with anti-Tollip antibody at 4° for 2 houts. The immunoprecipitates were analyzed by Western Blot analysis with inicated antibodies.
3.5.3.3 Rab5 and Rab7 interact with IL-1R and with TRAF6, and not with Tollip directly

The Rab (Ras like in Rat Brain) GTPases are a large family proteins modified by geranylgeranyl modification allowing them to associate with membranes. Rab GTPases are involved in endocytic trafficking, endosome fusion and exocytosis. Rab GTPases are localized to the surface of specific organelles of both endocytic and exocytic pathways. For example Rab4, Rab5 and Rab11 are localized to early endosome, Rab7 and Rab9 localize to late endosome and lysosomes.

Like other GTPase, Rab proteins cycle between an active form, that is GTP bound, and inactive state, that is GDP bound. In the active state, they regulate multiple steps in vesicular membrane trafficking of transport vesicles and cargo membrane proteins. Rab5 is a early endosomal protein, which interacts with SNARE complex recruiting EEA1 to regulate endosome motility and fusion, as well as clathrin coated-vesicles. Rab7 is involved in lysosomes biogenesis and in lysosomal sorting from late endosomal compartments.

The ability of Rab5 and Rab7 to interact with IL-1RI, Tollip and TRAF6 was tested. HEK293T cells were cotransfected with GFP-Rab5 and with Flag tagged TRAF1 to 6, to perform immunoprecipitation with Flag antibody. Western Blot analysis of the immunoprecipitated Flag-TRAFs or GFP-Rabs showed a strong interaction of GFP-Rab5 and GFP-Rab7 with TRAF6 and weak interaction with TRAF2, 3 and 5 (Fig. 2.21a and c, data not shown). In addition, GFP-Rab5 or GFP-Rab7 also interacts with Flag IL-1R chains and Flag-TRAF6 (Fig. 2.21b), but not with VSV-Tollip (Fig. 2.21d). Interestingly, we observed that GFP-Rab5 and 7 are highly modified when coexpressed with TRAF6 and not with IL-1Rs (Fig. 2.21b).

These preliminary results suggest the interesting possibility that TRAF6, and perhaps others TRAFs, is involved in endocytosis in addition to its role in signaling. TRAF6 could regulate internalization of IL-1RI and coated pit formation via Rab5, or participate in the late endosome sorting for lysosomal degradation via Rab7 (Fig. 2.21e).
Figure 2.21 IL-1Rs and TRAF6 interact with Rab5 and Rab7.
a. HEK 293T cells were cotransfected with Flag Tagged TRAF1-6 with GFP-Rab5. Cell extracts were prepared and immunoprecipitated with flag antibody, followed Western Blot analysis. b. HEK 293T cells were cotransfected with Flag Tagged TRAF6 or IL-1RI and IL-1RAcP with GFP-Rab5 or GFP-Rab7. Cell extracts were prepared and immunoprecipitated with flag antibody, followed Western Blot. c. HEK 293T cells were cotransfected with Flag Tagged TRAF1-6 with GFP-Rab5 or GFP-Rab7. Cell extracts were prepared and immunoprecipitated with GFP antibody, followed Western Blot analysis. d. HEK 293T cells were cotransfected with VSV Tollip with GFP-Rab5 or GFP-Rab7. Cell extracts were prepared and immunoprecipitated with VSV antibody. All Western Blotting were performed with indicated antibodies. e. Model of Rab 5 and Rab7 interacting with TRAF6 in Il-1R trafficking.
4. Conclusions

In summary, we observed that Tollip is ubiquitinated and this ubiquitination requires the presence of the CUE domain. Moreover Tollip’s CUE domain is required for high affinity binding of Tollip to ubiquitinated IL-1R during IL-1β signaling. We also showed that Tollip is an endocytic adaptor and that its CUE domain is an Ub–receptor involved in IL-1R endocytosis. Tollip is not necessary for IL-1RI internalization, but is required for IL-1RI normal trafficking from the late endosome to the lysosome for efficient degradation. Our results suggest that others Tollip interacting proteins, such as Tom1 and TRAF6, could mediate the function of Tollip in IL-1Rs endocytosis and in IL-1Rs signaling.

5. Discussion and perspectives

**Tollip CUE domain is a Ub-receptor for ubiquitinated proteins**

We showed that Tollip’s CUE domain acts as a Ub-receptor, able to bind monoubiquitin or polyubiquitin chains, as shown for other CUE domain containing proteins. We showed that Tollip’s CUE domain binds ubiquitinated IL-1RI. Therefore, it is possible that Tollip’s CUE domain may bind other TIR-containing receptors (i.e. TLRs) or ubiquitinated proteins. However specificity was shown by the fact that Tollip does not interacts with other ubiquitinated RTK receptors, like EGFR (Brian Brissoni, unpublished data). Proteins binding Tollip’s CUE domain could be ubiquitinated intermediates of ubiquitination pathways, such as E2 or E3 ubiquitin-ligases, that catalyze or promote cis-ubiquitination or trans-ubiquitination of proteins (i.e. TRAF6), as well as ubiquitinated endocytic adapters that define the localization of Tollip along the endocytic pathway or to regulate trafficking mechanisms of ub-IL-1R cargo (i.e. Tsg101, Tom1, Hrs).

**Tollip’s C2 domain and endocytic pathways**

We did not study the role of the Tollip’s C2 domain, but its features indicate a potential role in IL-1RI endocytosis and endosomal localization. The Tollip’s C2 domain was shown to bind phosphoinositols. Phosphoinositols levels are crucial for biogenesis and dynamics of endosomal vesicles. These phosphoinositols are localized to early and late endosomes and are recognized by FYVE domain found in certain endocytic adapters (i.e. EEA1, Hrs). The C2 domain of Tollip could likely act as phosphoinositol-binding motif, in alternative to FYVE domain, for Tollip localization to endosomal compartments.
. Further experiments using Tollip KE mutant or Tollip deficient cells reconstituted with the Tollip KE mutant should be useful to investigate the role of Tollip’C2 in endosomal localization. In addition, Tollip’s C2 ability to bind phosphoinositol is important for its inhibition of LPS induced NF-κB signaling as shown by overexpression of a Tollip C2 domain mutant unable to bind phosphoinositol (Tollip KE mutant)\(^{115}\). Therefore, it could be possible that Tollip’s C2 domain, regulating Tollip and IL-1RI endocytic processes, also influences TLR/IL-1R signaling.

**The nature and role of Tollip cis-ubiquitnation**

We showed that Tollip is modified by ubiquitin, and that its CUE domain is required for this ubiquitination. Our experiments suggested that Tollip is conjugated by ubiquitin polychains. However the exact topology of ubiquitin attached to Tollip remains unknown. We can hypothesize, due to the apparent stability of Tollip protein, that Tollip is not conjugated by K48-linked ubiquitin polychains, but rather to K63 or other lysine linked polychains. From our experiments, we cannot exclude that Tollip could be modified on multiple lysines or conjugated by both poly and monoubiquitin chains.

To determine the topology of Tollip ubiquitination, Mass Spectrometry sequencing (MS-MS) should be performed following isolation of His-Tollip protein under denaturing conditions (i.e. 6M guanidine), or by pull down of His-Tollip in the presence of Ubiquitin mutants (Ub\(^\text{K}^0\), Ub\(^\text{K48}\), Ub\(^\text{K63}\)).

Tollip ubiquitination is not regulated in a signal dependent manner, but is constitutive, thus suggesting a putative role for Tollip ubiquitination as a “third” docking domain. It could participate to the recruitment of other Ub-receptors involved in ubiquitination (i.e. E2, E3 ligase) or endocytosis (i.e. Tom1, Tsg101, Hrs). In addition, Tollip ubiquitination could have several other functions. These may include inhibition of the CUE domain of Tollip as Ub-receptor or other Ub-receptors (i.e. TAB2 and TAB3, Tom1, Hrs). In facts, Tollip cis-ubiquitination may block the access of its CUE domain or other ubiquitin-binding domain to other ubiquitin or ubiquitinated proteins. Finally, Tollip ubiquitination may modulates the cellular localization in the endosomal compartments. This hypothesis is supported by observations showing partitions of Tollip between cytosolic and membrane fractions. It has to be noted that endogenous ubiquitination of Tollip is technically very difficult to detect by immunoprecipitation procedure. This could be explained by the fact that only membrane-associated Tollip is highly ubiquitinated, so less accessible for immunoprecipitation.

**The Tollip phosphorylation?**
Our results in 2D SDS-PAGE suggest that, in addition to ubiquitination, Tollip may also be modified by phosphorylation. This phosphorylation, together with ubiquitination, could be an important mechanism for rapid regulation of Tollip function after signaling or during endocytic processes. To assess the role of phosphorylation of Tollip in signaling, endocytosis and cis-ubiquitination, further 2D SDS-PAGE experiments could be required to follow phosphorylation after IL-1β and LPS stimulation. In the same idea, overexpression studies of Tollip mutants, that no longer phosphorylated, could also be performed to investigate the role of Tollip’s phosphorylation.

**Ubiquitination and endocytosis of IL-1Rs and TLRs**

The IL-1R is the receptor for IL-1β, a potent inducer of inflammation, and its activity should be highly regulated to avoid negative effects of sustained signaling. Due to the efficiency of IL-1β signaling compared to the few copies of receptor at cell surface, the signal may be regulated by IL-1R endocytosis. We show that, after IL-1β stimulation, IL-1RI is monoubiquitinated, internalized and trafficked in the endosomal compartments followed by degradation after a few hours, as shown for other ubiquitinated receptors (i.e. EGFR, IL-2R). In this way the cell could regulate by Endocytosis and degradation the fade the receptor from the cell surface, to block signal from IL-1RI. It is also possible that the IL-1RI internalization is also necessary for optimal response in the time after internalization, or in the space for signaling form the endosome, as is the case of RTK (i.e. EGFR, NGF). Therefore endosomal internalization of the ubiquitinated receptors mediated by Tollip may be also required for the formation of signaling complexes inside those organelles, as it is the case for some TLRs (i.e. TLR9, TLR7).

In contrast to other ubiquitinated receptors IL-1RI requires the association with co-receptor during the signaling. We observed that both IL-1RI and IL-1RAcP are ubiquitinated but to different extents. Therefore, it is possible that also IL-1RAcP is targeted for trafficking and degradation. It is probable that, Tollip as IL-1RAcP interacting protein, may binds more to this chain because more ubiquitinated.

Previous observations have shown that Tollip binds TLR2 and TLR4 and is a key factor in TLRs signaling, as revealed by Tollip deficient mice characterization (see chapter 1). These findings, together with preliminary results indicating that TLR4 and TLR2 are ubiquitinated like IL-1R, (Brian Brissoni, unpublished data) raised the hypothesis that TLR2 and TLR4 could also be regulated by endocytosis mediated by Tollip.
**IL-1Rs monoubiquitination**

The IL-1Rs is multiply monoubiquitined, as observed for EGFR \(^1\). As for EGFR, the presence of multiple ubiquitin moieties on IL-1RI increases the affinity for Ub-receptor domains of endocytic adapters. In addition, monoubiquitination of the receptors could also act as a regulated amplification signal for cis- and trans-ubiquitination of other proteins. It is possible that multiple monoubiquitin around the IL-1Rs recruit ubiquinating enzymes that promote the ubiquitination of a network of other proteins necessary for the endocytic processes. This phenomenon is observed around activated RTK where endocytic adaptors (i.e Epsin, Eps15, Hrs) are monoubiquitinated during the endocytosis \(^2\).

Experimental approaches using MS-MS sequencing of purified IL-1Rs could be used to map ubiquitinated lysines and to discover new proteins involved in IL-1β triggered IL-1R ubiquitination (E2/E3 ligase, kinases) and/or in its endosomal trafficking.

**Tollip as endocytic adaptors for late endosome trafficking of IL-1RI**

The late endosome sorts proteins for degradation by the formation of an internal membrane, which fuses with the lysosome. The luminal membrane vesicles of late endosome are distinct from the external limiting membrane in its lipid composition (enriched in cholesterol, phosphoinositol, LBPA) \(^3\). Here we have shown that a consistent pool of Tollip localize to the late endosome. However, it was not possible to determine exactly if Tollip localizes in the external or internal membranes using regular costaining with LAMP or LBPA. This question could be addressed by electron microscopy. Consistent with this first observation, the accumulation of IL-1R cells in large LAMP positive vesicles (late endosomal/lysosomal marker) of Tollip deficient cells strongly suggests that the endocytic function of Tollip for IL-1R trafficking occurs between the late endosome and the lysosome. In the late endosome Tollip and its ubiquitinated IL-1Rs cargo, may interact with several endocytic proteins (see next paragraph) to control the lysosomal targeting of the ubiquitinated-cargo, to regulate the translocation to internal vesicles of late endosome, for subsequent sorting to the lysosome.

**Tollip in a multiprotein complex with endocytic proteins**

Tollip may bind several endocytic proteins (i.e. Vps23/Tsg101, Vps28, Hrs) associated with early and late endosomes for ubiquitinated-cargo sorting to lysosomes. It is possible that Tollip could form a complex web of interactions with several endocytic and signaling proteins, as pitted in figure 2.22.

Our results showed that Tom1/Tom1L1, an endocytic adapter interacting with Tollip, could
mediate the endocytic function of Tollip in IL-1Rs trafficking. Tom1 was shown to be localized in early endosome \textsuperscript{218,221}, where it could recruit Tollip and Ub-IL-1Rs to allow trafficking to late endosomes. Tom1 may by interact initially in the early with Tollip and ubiquitinated IL-1RI to participate to the trafficking from early endosome to late endosome, as well targeting for lysosomal degradation. This hypothesis is also supported by the observation that Tom1 overexpression induces low level of IL-1Rs by Western Blot, probably by an enhancement of degradation of IL-1Rs, suggesting a role of Tom1 with Tollip to drive IL-1R lysosomal degradation. To confirm this hypothesis, several control experiments should be carried out in control and Tollip deficient MEFs using with protease inhibitors (i.e. Leupeptin), lysosomal degradation inhibitor (i.e. chloroquine) or proteosomal inhibitors (i.e. Lactacystin, MG132) (in progress).

Tom1/Tom1L1 was also found to interact with ubiquitinated proteins and several others endocytic proteins, such as clathrin, endofin, as well as Hrs and Tsg101 \textsuperscript{217}. Tsg101 and Hrs bind ubiquitin via their ubiquitin binding domain, and both are endocytic adapters important for late and early endosome trafficking \textsuperscript{162}. Tsg101 is a UEV-containing protein with E2 ligase function and is a member of the ESCRTI proteins complex (FYVE-PI3P binding domain) \textsuperscript{233,234}. Tsg101 is involved in the ubiquitin-cargo sorting to the Late endosome and viral budding\textsuperscript{235}. Hrs was shown be a endocytic adaptor for EGFR internalization and degradation, and binds to the ESCRTI complex via Tsg101 and clathrin coated vesicles \textsuperscript{193,236}. These findings, together with our observations, suggest that Tollip and its ubiquitinated IL-1RI cargo may interact with Tom1/Tom1L1 along the endocytic pathway and potentially with several other endocytic adapters to form a large complex responsible for the trafficking and endosomal fate of IL-1RI (Fig. 2.22). To confirm the interaction between with IL-1Rs, Tollip and Tom1 with Tsg101 and/or Hrs, immunoprecipitation in overexpression systems and confocal colocalization studies are indeed required (in progress).

We also showed TRAF6 binds Tollip, forming a complex after stimulation. TRAF6 can act as a platform not only for signaling proteins, but also for endocytic proteins, suggesting a novel function of TRAF6 in endocytic processes. This hypothesis is supported by the fact that Rab5 and Rab7 bind to IL-1RI and TRAF6. The Rab complex with TRAF6 or the receptors could regulate vesicles dynamics in the endosomal compartments during IL-1R trafficking.
Figure 2.22. Model of Tollip interactions in the IL-1R/TLR signaling and endocytic pathway.
Concluding Remarks

Tollip (Toll-interacting protein) was originally identified through a yeast-two-hybrid screen using the IL-1RαCp. Subsequent studies showed that Tollip is also able to interact with several other members of the TIR superfamily members, including TLR2 and TLR4. Overexpression of Tollip results in inhibition of TLR2- and TLR4-mediated NF-κB activation. Tollip interacts with IRAK1, and the level of IRAK1 autophosphorylation is decreased in the presence of Tollip. After TLR stimulation, IRAK1 causes phosphorylation of Tollip. Although the physiological importance of this is unclear, it is possible that phosphorylation of Tollip facilitates the ubiquitination of IRAK1 and its subsequent degradation. It is intriguing that Tollip phosphorylation by IRAK might lead to the release of Tollip from the Tollip-IRAK complex, and might overcome the negative regulation of Tollip. In addition, Tollip expression is elevated in intestinal epithelial cells, which are hypo-responsive to TLR2 ligands. So, phosphorylation and dephosphorylation of Tollip and IRAK in TLR signalling might be a switch for TLR4 and TLR2-mediated responses. The C2 domain in the N-terminus of Tollip, which might be involved in its inhibitory role. Li et al. showed that Tollip’s C2 domains is involved in binding various phospholipids, and preferentially binds to phosphatidylinositol-3-phosphate and phosphatidylinositol-3,4,5-phosphate. Mutation of a vital lysine residue (K150) to glutamic acid (Tollip K150E) within the C2 domain abolishes this binding, and the Tollip K150E mutant is unable to inhibit LPS-induced NF-κB activation, indicating that its lipid-binding capability is in someway connected to the inhibitory role of Tollip. It is currently not clear whether Tollip also affects other TLR signalling pathways, and the role of Tollip in disease remains to be explored. Tollip expression is elevated in intestinal epithelial cells (IECs), which are hypo-responsive to TLR2 ligands. This indicates that Tollip might negatively regulate TLR signaling in IECs, although no mechanism of action has been determined.

During this thesis work, we have tried to characterize the role of Tollip in the IL-1R/TLR signaling. The analysis of Tollip deficient mice showed that Tollip regulates the magnitude and kinetics of IL-6 and TNFα production upon stimulation with IL-1β and low doses of LPS. Surprisingly activation of NF-κB, JNK and p38 signaling appeared normal in Tollip −/− cells. This could easily be explained if Tollip deficiency resulted in subtile defects in the activation of the membrane proximal signaling complex (Tollip is rapidly and transiently recruited to this complex), and thereby ultimately in downstream activation of
these signaling cascades. These results suggest that Tollip play a role in the modulation of the IL-1R/TLR signaling, where it may regulate biochemical events (modifications, degradation, interaction).

We did not observe any effect of Tollip deficiency in response to lethal doses of LPS, suggesting that Tollip acts in ‘fine tuning’ or coordinating optimal signaling through IL-1RI and TLR4. Given that the physiological concentrations of IL-1β or LPS at the sites of infection/inflammation most likely do not exceed the low nanomolar range, Tollip is undoubtedly an important mediator of these responses. In this way, Tollip’s resembles a scaffolding protein that is required for optimal responses. As such, high concentrations of Tollip may titrate out critical signaling proteins in IL-1R and TLR4 pathways, thereby blocking signaling. This would accommodate the apparently conflicting observations (i.e. repressor vs. activator) and predicts that the cellular concentration of Tollip may be crucial for its capacity to regulate signaling. Future studies are now required to address the precise molecular mechanisms by which Tollip modulates cytokine production/secretion. Although we cannot exclude that Tollip does not contribute entirely or partly to the initial activation of NF-κB, JNK and p38 signaling, it is also possible that Tollip modulates signaling via alternative means. Several recent observations suggest that Tollip may participate in the recognition and trafficking of ubiquitinated proteins within endocytic/biosynthetic pathways. Endosomes, via associated proteins, can function as platforms for the formation and activation of specific signaling complexes. It thus conceivable that endosomally localized Tollip is involved in optimal responses to IL-1β and LPS.

With this work we also demonstrated that Tollip is a member of the endocytic pathway, due to some particular features as an adaptor, such as ubiquiting binding, cellular localization, phosphatidylinositol-phosphate binding. Tollip acts as an endocytic adapter which is specifically required for trafficking of IL-1RI from the late endosome to the lysosome and its efficient degradation, but not for all ubiquitinated receptors i.e. EGFR trafficked through late endosome to lysosome (Brissoni et al., manuscript in review).

We have shown that this protein and its CUE domain plays a role in the trafficking to lysosomal degradation of ubiquitinated cargo IL-1R, eventually also for TLRs. First, in Tollip deficient cells, IL-1RI is trafficked to LBPA positive vesicles, indicating that it is not crucial for internalization or sorting to the late endosomes and suggests that other upstream endocytic adapters are involved. Second, IL-1RI accumulates at the late endosome and is not efficiently sorted to the lysosome in the absence of Tollip. Third, reconstitution of deficient cells with
wildtype but not mutant Tollip, defective in ubiquitin-binding, reverses the accumulation of IL-1RI at the late endosome. Fourth, a major pool of Tollip colocalizes with Lamp1 and LBPA positive endosomes and the morphology of these endosomes appears normal in Tollip deficient cells. Fifth, Tollip function is localized in the late endosome where act together with Tom1 and others endocytic proteins (i.e. Tsg101, Hrs, Rab). Tollip is probably necessary for the recruitment of Tom1 to late endosomes and mediates a Tom1/IL-1RI interaction. The overexpression of Tom1, but not Tom1ΔGTA resulted in the accumulation of IL-1RI on late endosomes, suggesting that Tom1 also regulates sorting of IL-1RI and that its GTA domain which is required for it to reside on late endosomes plays an essential role (Brissoni et al., manuscript in reviews).

Interestingly, the Tom1 related protein, Tom1LI interacts via its VHS domain with the ESCRT component Tsg101. ESCRT mediates the formation and sorting of proteins into the lumen of the late endosome forming multivesicular endosomes (MVE), which ultimately fuse with lysosomes. It is therefore tempting to speculate that IL-1RI is passed from Tollip to Tom1/Tom1LI at the late endosome. IL-1RI-Tom1/Tom1LI complexes in turn interact with Tsg101, triggering incorporation of IL-1RI into the luminal of the late endosome from whereby it ultimately becomes exposed to the hydrolytic components of the lysosome. Further studies are now necessary using Tom1 and/or Tom1LI -/- cells to confirm their role in endocytic trafficking of IL-1RI and to identify other components of IL-1RI endocytic sorting machinery.

Finally it is important to mention that Tollip deficiency does not have obvious effects on signaling pathways (i.e. IkB degradation, JNK, p38, ERK phosphorylation was measured) in cells isolated from Tollip -/- mice, yet surprisingly resulted in decreased IL-1β or LPS induced TNFα and IL-6 production. Finnally, we showed that Tollip has a crucial role in the fine tuning of these signaling pathways acting as a “double edge sword”, inhibiting but also positively modulating the signal. Tollip is not essential for activation of the signaling, but is required to regulate the quality of the response (i.e. cytokines production). In another hand, Tollip was also shown to be upregulated to inhibit the signaling in the tolerance phenomenon following restimulation with these signals (i.e. IL-1β, LPS). This dual effect of Tollip on signaling is dependent on its expression levels in the cell, and could be important to regulate inflammatory and innate immune response during pathogen infections.

Therefore, we showed that Tollip is crucial for trafficking and degradation of the IL-1RI in the lysosomes. Growing evidence indicates that an important interplay exists between
endocytosis and signaling. It will now be pertinent to understand how Tollip (and in general endocytic adapters) assemble endocytic machinery around trafficking cargo and serve as scaffolds for signalosomes, as modulation of this cross-talk may provide an effective means of regulating IL-1β-triggered inflammation. Future studies are now required to explore the links between receptor signaling and ubiquitin mediated regulation of IL-1RI/TLRs by Tollip. It could be predicted that the progressive accumulation of receptors in the late endosome will ultimately inhibit the important interplay that exists between signaling and endosomal/membrane biogenesis. The functional consequences of this could thus touch the multiple roles of this organelle in endocytosis, phagocytosis, cholesterol trafficking, and thereby exacerbate numerous diseases (i.e. certain cancers, lung and vascular disease) that can be attributed to alterations in endocytic trafficking.
Figures acknowledgments:

**Chapter 1:** Also participates at experiments in the following figures

- Arnaud Didierlaurent and Jean-Claude Sirard: Figures: 1.5
- Arnaud Didierlaurent: Figure 1.6, 1.7, 1.8

**Chapter 2:** Also participates at experiments in the following figures

- Kimberly Burns & Natalia Aebi: Figures: 2.4, 2.10, 2.11, 2.19, 2.21
- Kimberly Burn, Laetitia Agostini: Figure 2.12 to 2.14
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Publications

CARMA1 is a critical lipid raft-associated regulator of TCR-induced NF-kappa B activation.

Inhibition of interleukin 1 receptor/Toll-like receptor signaling through the alternatively spliced, short form of MyD88 is due to its failure to recruit IRAK-4.

Tollip is an endocytic adapter that regulates lysosomal trafficking of the IL-1RI.
Submitted

Tollip regulates Pro-Inflammatory Responses to Interleukin-1 and lipopolysaccharide.
In press, MCB.

Languages & Skills

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