CHARACTERIZATION OF BIOLOGICAL FUNCTION OF INTERACTION BETWEEN TLR4 AND PLIC-1

by

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Toll-like receptors (TLRs) are key innate immune receptors that recognize non-self pathogens and trigger host responses. Activation of these receptors results in the release of antimicrobial peptides, inflammatory cytokines, and co stimulatory molecules that initiate adaptive immunity. For infections with gram-negative bacteria, lipopolysaccharide is the main source of inflammation, and toll-like receptor 4 (TLR4) is crucial in mediating its effects. TLR4 is expressed on cardiomyocytes, macrophages, airway epithelia, endothelial, smooth-muscle cells and in small amounts in most other tissue. But, uncontrolled activation of TLR signaling molecules may cause auto immune diseases, sepsis, and tissue damage so the activation of TLR4 should be under control. Ubiquitin–dependent receptor degradation as well as stabilization was recently suggested as a novel regulatory mechanism in controlling several TLR activations. We have recently found that an ubiquitin-like protein named protein linking integrin associated protein to cytoskeleton 1 (PLIC-1) interacts with the cytoplasmic domain of TLR4. The interaction between TLR4 and PLIC-1 was verified by western blot and immunoprecipitation. Further mapping of the interacting domain was done and we observed that the N terminal fragment of PLIC-1 is interacting with TLR4. PLIC-1 has been reported to stabilize proteins by interfering with proteosomal degradation. Consistent with this finding, we observed that over expression of PLIC-1 accumulated ubiquitinated TLR4. By flow cytometric analysis we observed that over expression of PLIC-1 is stabilizing TLR4. Reporter studies show that PLIC-1

inhibits the TRIF-dependent IFN-β pathway. When endogenous PLIC-1 was knocked down by RNAi, the activation of TRIF-dependent IFN-β luc was further increased. The same effect was observed in J774 mouse macrophages. Taken together our results suggest that PLIC-1 is a negative regulator of TLR pathway. This knowledge may be applied in immunotherapy as a means to modulate TLR activation in diseases such as septic shock, thus provides benefit for public health.

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1.0 INTRODUCTION

1.1 Toll Like Receptors:

The immune system of vertebrates can be divided into two categories: the innate immune system and the adaptive immune system. The innate immune system exists to provide early defense against pathogen attack and to alert the adaptive immune system to the fact that pathogen invasion has begun. Toll like receptors (TLRs) are critical innate immune receptors that recognize microbial pathogen and trigger the first line of host defense [1-5]. The name Toll appeared in the literature in the early 1980s and was given to one of the mutants found in a *Drosophila* genetic screen. It was first described as an essential molecule involved in fly development. It was shown by Lemaitre et al that mutant Drosophila carrying loss-of-function mutation in the Toll receptor results in high susceptibility to fungal infection [6]. To date 10 different TLRs have been identified in humans and 13 in mice [7]. Toll like receptors are critical innate immune receptors that recognize microbial pathogens and trigger the first line of host defense. They are mainly expressed on antigen-presenting cells, such as macrophages or dendritic cells, and their signaling activates antigen-presenting cells to provoke innate immunity and to establish adaptive immunity [8]. Different Toll like receptors recognize different molecular patterns in pathogen, showing that they have different ligands. TLR1, TLR2 and TLR6 recognize various bacterial components like peptidoglycan, lipopeptide, and lipoprotein of Gram positive bacteria [9-13]. During replication in the host many viruses produce double

stranded RNA which is recognized by TLR3 [14]. LPS of Gram negative bacteria are recognized by TLR4 [15]. TLR5 recognizes bacterial flagellin [16] and TLR7 recognizes synthetic imidazoquinoline-like molecules, guanosine analogs such as loxoribine, and single stranded RNA (ssRNA) derived from human immunodeficiency virus type-1 (HIV-1) [17-19]. TLR9 functions as a receptor for bacterial DNA containing CpG motifs [20-21].

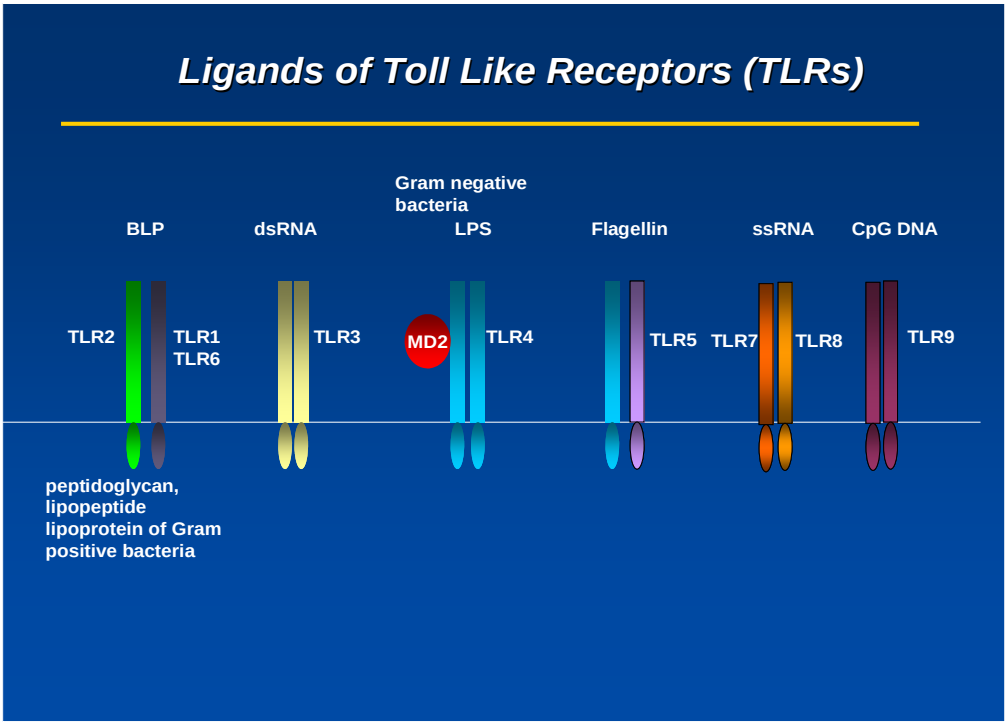


Fig 1: Ligands of different Toll Like Receptors (TLRs)

1.2 Toll Like Receptor 4 (TLR4):

Toll Like receptor (TLR4) is well known for its sensitivity to Bacterial Lipopolysaccharide (LPS). LPS is found in Gram negative bacteria and is a potent activator of the innate immune response in humans. Bacterial LPS is a cell-wall component of gram-negative bacteria that has the ability to induce a dramatic systemic reaction known as septic shock. LPS-binding protein

(LBP), and the receptor protein CD14, binds to LBP-bound LPS. Both LBP and CD14 carry leucine-rich repeat motifs [22]. When TLR4 binds to CD14 complexed with its LBP:LPS ligand, recognition of LPS occurs largely by the mammalian LPS receptor — the TLR4−MD2−CD14 complex — which is present on many cell types including macrophages and dendritic cells [23-24]. CD14 concentrates LPS for binding to the TLR4−MD2 complex. TLR4 recruits some adapter protein molecules in the cytoplasm, which eventually activates the transcription factor NFκB and inflammatory cytokines like IFN-β in the nucleus.

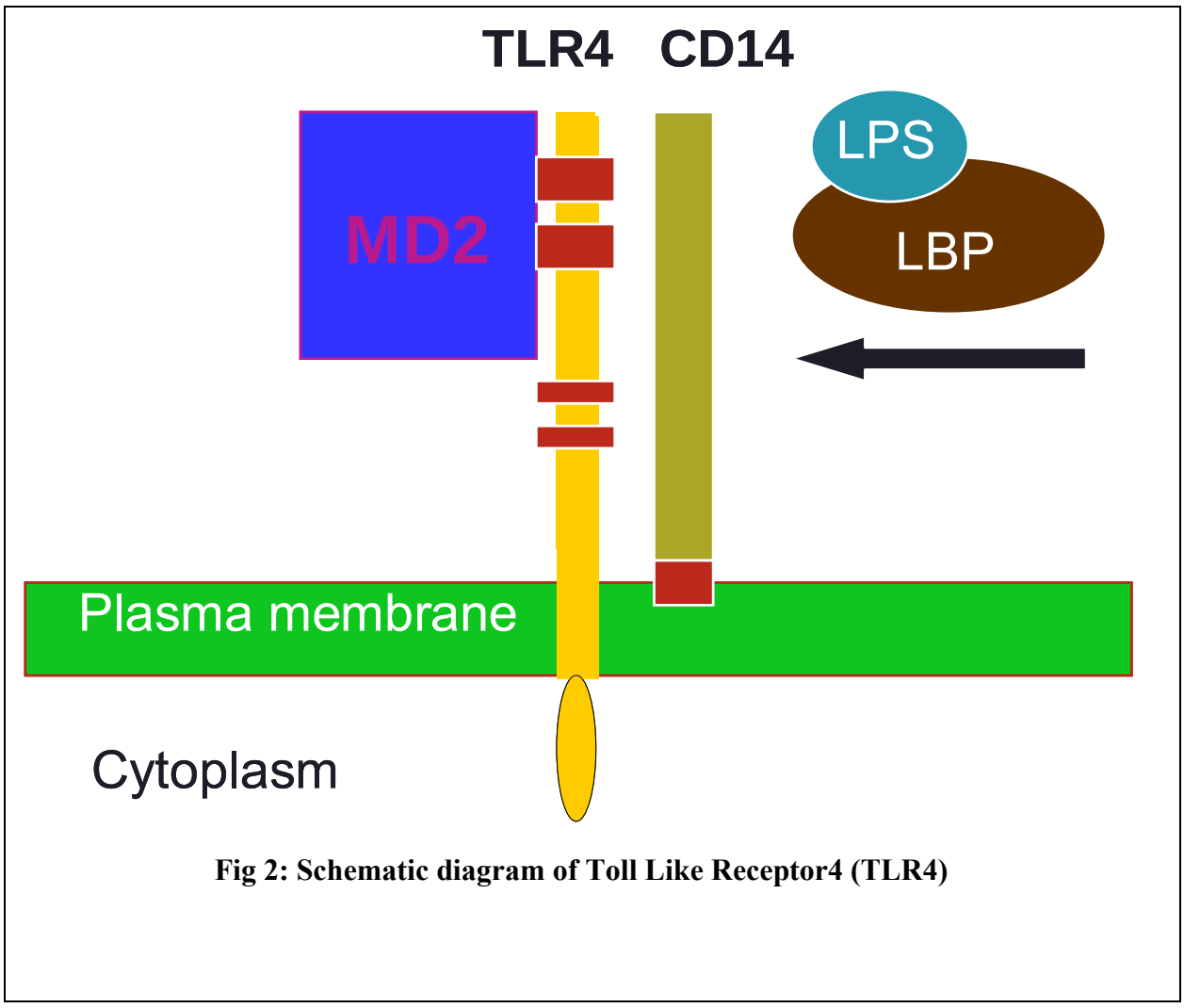


Fig 2: Schematic diagram of Toll Like Receptor4 (TLR4)

1.3 TLR4 signaling pathway:

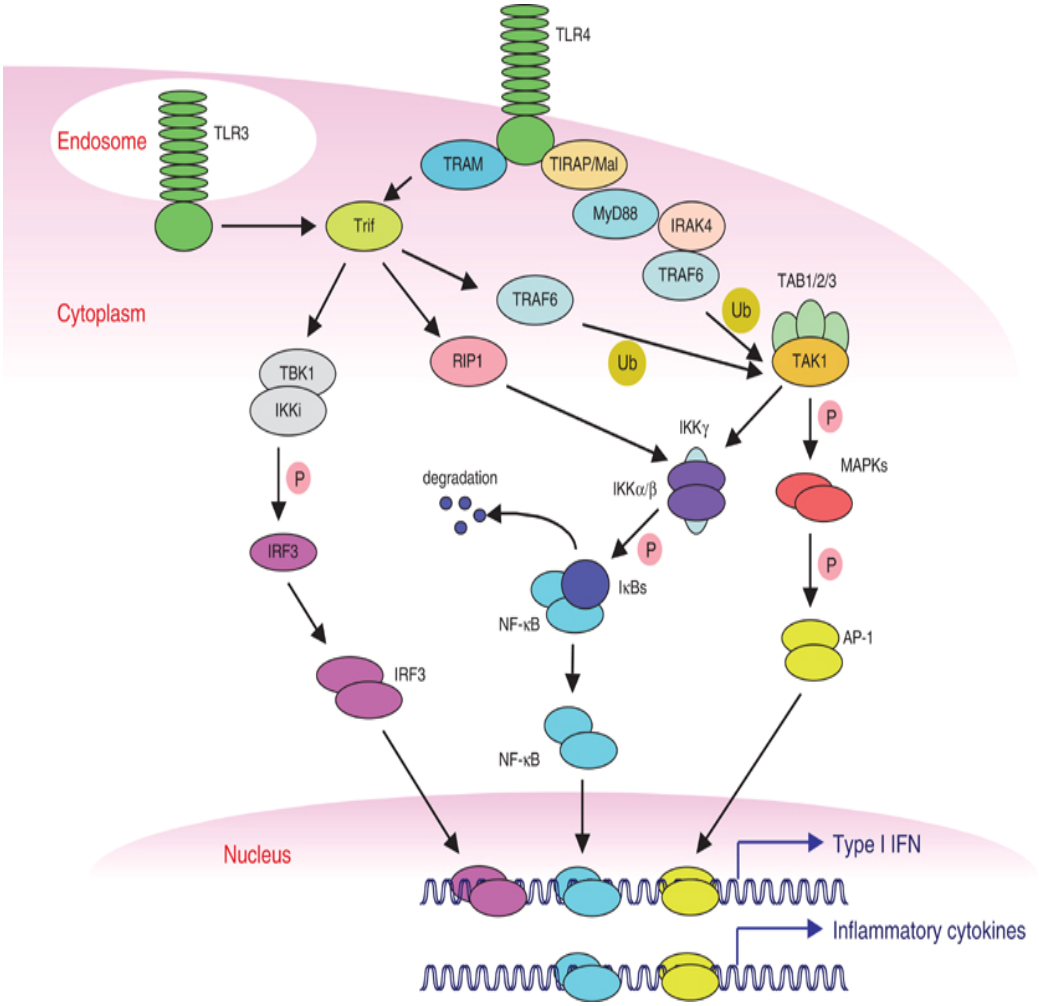
MyD88-dependent pathway:

Toll like receptors recognize pathogen and initiate immune response to eliminate invading pathogens. A key structural motif which is involved in the signal transduction is the Toll/IL-1 receptor (TIR) domain [25]. The extra cellular domain of the Toll Like Receptor structure, rich in leucine residues is termed the leucine-rich repeat and is involved in ligand recognition [26]. The intracellular region contains a structure common to TLR and IL-1 receptor family members, called the Toll/IL-1 receptor homologous (TIR) domain, which is essential for signal transduction. The proximal events of TLR-mediated intracellular signaling are initiated by TIR-domain-dependent heterophilic interactions with TIR-domain-containing cytosolic adapters such as myeloid differentiation primary response protein 88 (MyD88). MyD88 was first found to be critical for TLR signaling [27]. MyD88 has death and TIR domains at its N- and C-terminal portions, respectively. The TIR domain is involved in interaction with TLRs, and the death domain recruits IL-1 receptor– associated kinase (IRAK) family members [28]. The association of TLRs and MyD88 recruits members of the interleukin-1 receptor-associated kinase (IRAK) family. So far, four IRAKs are identified: IRAK1, IRAK2, IRAK4 and IRAK-M. IRAK1 and IRAK4 possess intrinsic serine/threonine protein kinase activities, but IRAK2 and IRAK-M lack this activity, IRAK-M deficient cells consistently show hyperproduction of inflammatory cytokines in response to various TLR ligands [29]. MyD88 activates the serine/threonine IRAK kinases IRAK1 and IRAK4. The IRAKs then enable the recruitment and activation of tumor necrosis factor receptor-associated factor 6 (TRAF-6), a RING-domain ubiquitin ligase that activates the TAK1 kinase through polyubiquitination [30]. TAK1 in turn activates the IKK complex, which phosphorylates IκBs (NF-κB inhibitors) and targets them for ubiquitination and

subsequent degradation. This releases NF-κB, which moves to the nucleus and regulates its target genes, including those that encode proinflammatory cytokines [31].

MyD88-independent pathway:

The MyD88-independent pathway was first revealed by the retained response of MyD88-deficient mice to LPS. TLR4 signaling can activate NF-κB and MAPKs in MyD88-deficient cells [32]. These delayed responses fail to induce gene expression of inflammatory cytokines but are sufficient for DC maturation, or expression of IFN-β and IFN-inducible genes. IFN-β gene expression requires IRF-3 activation, which is detected in LPS activated MyD88-deficient macrophages. TLR4 requires another adapter protein which is TRIF related adapter molecule and also known as TICAM-2, for the association with TRIF. TRIF can interact with the IκB kinases IKKe and TANK-binding kinase 1 (TBK1). IRF-3 is phosphorylated by these kinases, translocates to the nucleus, and induces several target genes, including IFN-β. TRIF can also lead to NF-κB activation through TRAF6 [33]. Furthermore, TRIF can also interact with receptor-interacting protein (RIP) 1 through an RIP homotypic interaction motif. This interaction is also critical for NF-κB activation. NF-κB activation leads to the expression of IFN-β and inflammatory cytokines.

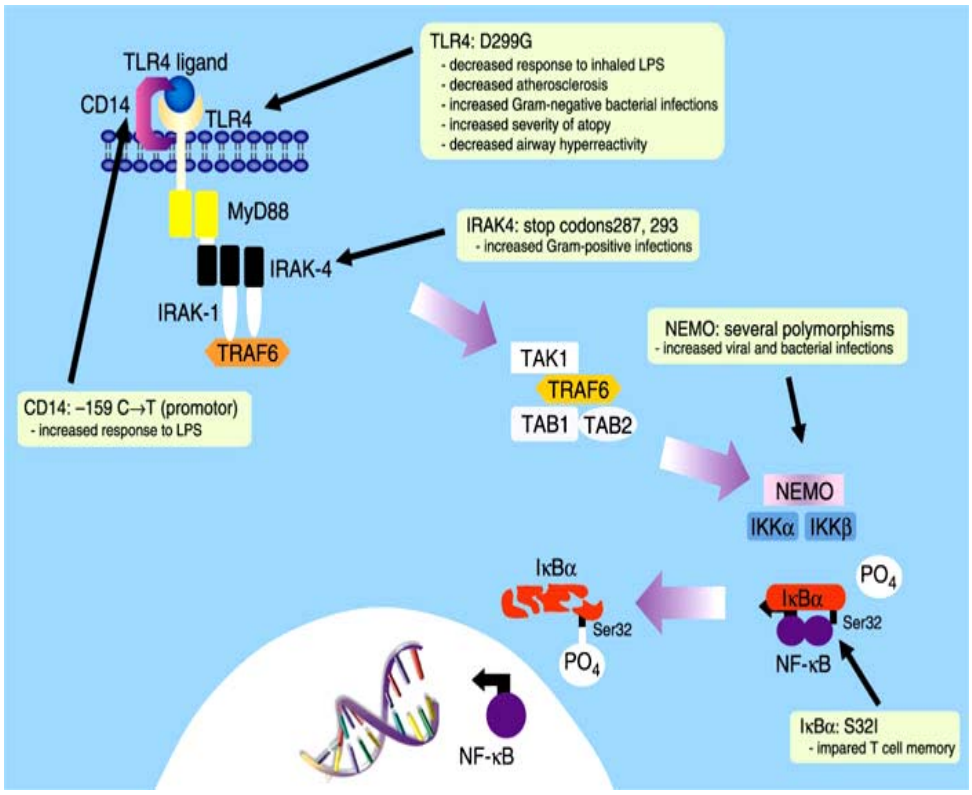


Cell Death and Differentiation (2006) 13, 816–825.

Fig 3: TLR4 signaling pathway

1.4 Why Toll Like Receptors are important:

Studies of people with specific polymorphisms in genes encoding TLRs or their downstream signaling molecules can elucidate relationships between TLR signaling and human disease. Until now the best-studied TLR polymorphism is an amino acid substitution, from aspartic acid to glycine at position 299 (D299G), in TLR4 [34]. This polymorphism causes decreased airway response to inhaled bacterial lipopolysaccharide (LPS) in humans and a corresponding decreased signaling response to LPS. Sepsis and its most severe form, septic shock, represent a syndrome associated with bacterial infection caused by Gram negative bacteria. Because TLR4 is required for the innate immune response to Gram-negative LPS in mice, several groups have studied the effect of the D299G polymorphism on humans at risk for sepsis. This also causes Gram-negative infections [35] and systemic inflammatory syndrome [36]. Polymorphisms in IRAK-4 are also associated with impaired responses to bacterial infection. These polymorphisms include single-nucleotide changes that encode stop codons at amino acid positions 287 and 293 of IRAK-4. Children homozygous for these polymorphisms have recurrent infections, caused mostly by the Gram-positive bacteria *Streptococcus pneumoniae* and *Staphylococcus aureus* [37]. Mutations in the gene encoding NEMO give rise to anhidrotic extodermal dysplasia with immunodeficiency. Patients with this disease present with sparse hair, anhidrosis and severe bacterial infections [38]. It was also observed by Kiechl *et al* that polymorphisms in human TLR4 might facilitate these genetic approaches, particularly for cardiovascular disease. Thus, the D299G polymorphism is associated with a reduced risk for carotid artery atherosclerosis [39].



Schwartz, et.al Nature Immunol. 5, 975-979 (2004)

Fig 4: Association of polymorphisms of TLR signalling molecules with human diseases.

1.5 Negative regulation of Toll signaling:

Several regulatory mechanisms that seem to control TLR signaling have been described. It was shown that IRAK-M prevents the dissociation of IRAK4 and IRAK1 from MyD88. In response to stimuli, IRAK4 and IRAK1 are sequentially phosphorylated, which results in activation of tumor necrosis factor receptor-associated factor 6 (TRAF6) [40]. TRAF6 activates transforming growth factor-β-activated protein kinase 1 (TAK1), a member of the MAP kinase kinase kinase (MAP3K) family, in a ubiquitin-dependent manner. TAK1 activates the IKK complex that leads

to NF-κB activation. Thus IRAKM here functions as a negative regulator of the Toll signal pathway. Production of inflammatory cytokines in response to various TLR ligands is ablated in IRAK4-deficient mice. Recently it was found that Triad3A, an E3 ubiquitin-protein ligase enhances ubiquitination and proteolytic degradation of some TLRs [41]. The soluble form of orphan receptor ST2 (also known as T1) binds directly to myeloid lineage cells and downregulates expression of TLR1 and TLR4 [42].

1.6 Protein Linking Integrin associated protein to Cytoskeleton 1 (PLIC-1):

PLIC-1 is a type 2 Ubl (Ubiquitin like) protein which has a wide variety of cellular functions. It inhibits the cell cycle to stabilize plasma membrane proteins and also to rearrange the cytoskeleton [43]. There are four members of PLIC-1 in mouse and human. The PLIC proteins belong to a family of proteins that contain an ubiquitin-like (ubl) domain as an integral part of their open reading frame [44]. As reported by Tanaka, et al in 1998, ubiquitin-like (ubl) proteins can be subdivided into two general classes; small-sized type 1 ubl proteins that are covalently linked to target proteins in a fashion similar to ubiquitin, and type 2 ubl proteins that are not ligated to other proteins and whose functions are not yet well understood [45]. hPLIC-1 and hPLIC-2 are examples of type 2 ubl proteins. Functional data suggest a role for the hPLIC proteins in the *in vivo* degradation of several proteins known to be ubiquitination-dependent substrates of the proteasome [46].

1.7 General biology of ubiquitin and ubiquitin like proteins:

Ubiquitin is a 76 amino acid globular protein that is nearly identical throughout eukaryotes [47]. Ubiquitination, the process of conjugating ubiquitins to other proteins is fundamental for degradation of protein whose level is regulated either constitutively or in response with external

stimuli [48]. The enzymes involved in ubiquitination have been extensively studied and reviewed. Ubiquitin is first activated by ubiquitin activating enzyme known as enzyme 1 or E1 and then transferred to a ubiquitin conjugated enzyme, Ubc or E2 that together with a ubiquitin protein ligase E3, covalently attaches ubiquitin to the target protein at the amino acid group of a lysine residue [49]. The polyubiquitin chain is elongated by E2 and E3, sometimes with the help of accessory factor E4. By forming peptide bonds between the C-terminal glycine of ubiquitin and the lysyl amino group of the ubiquitin that has previously been added to the chain, polyubiquitination signals the 26S proteosome to degrade the ubiquitylated proteins[50]. It was reported that PLIC-1 inhibits the degradation of ubiquitinated protein and from the experiments we performed it was clear that PLIC-1 is accumulate more ubiquitinated protein including TLR4.

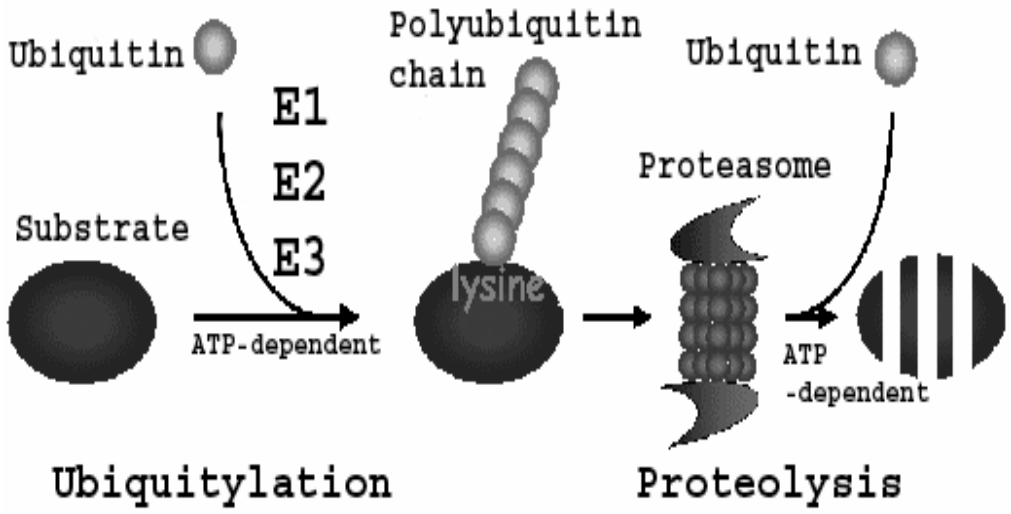


Fig 5: Polyubiquitination mediated proteolysis

2.0 MATERIALS AND METHODS

2.1 Cell Lines and reagents:

The 293T cell line ATCC CRL-11268 was cultured in DMEM (Invitrogen Life Technologies) supplemented with 10% FBS (HyClone), 1% penicillin, and 10 μg/ml streptomycin. The mouse macrophage cell line J774 was a generous gift from Dr David Hackam (University of Pittsburgh School Of Medicine, Department Of Surgery, Pittsburgh PA).

2.2 Plasmids:

PLIC-1 N terminal and PLIC-2 full length plasmid was a kind gift from the Howley lab (Boston). The IFN NF-κB LUC plasmid was obtained from Drs. J. Pomerantz and M. Boldin (Caltech, Pasadena, CA).YFP-TLR4, Flag-TLR4, HA-PLIC-1, YFP-PLIC-1, Flag PLIC-1, Fc-TLR4 and MIR-DFT-empty plasmid, YFP TRIF, Flag TRIF plasmids were constructed in the lab. Myc Triad3A and YFP Triad3A plasmids were a kind gift from Dr. Ulevitch lab, Scripps Research Institute, San Diego. pCL-10A1 which is a retrovirus packaging vector was obtained from IMGENEX. RNAi constructs targeting human endogenous PLIC-1, 1602, 733, 819 and LMP PLIC-1 and RNAi targeting mouse endogenous PLIC-1, m LMP were constructed in the lab previously.

2.3 293T Transient transfection:

Transfection was done with Lipofectamine 2000 (Invitrogen Life Technologies) transfecting reagent and the amount of plasmid indicated. Plasmids and media (amount depending on the size of the well) was mixed together, and lipofectamine 2000 (double the amount of plasmid) was mixed with media in a separate tube. Then media containing plasmid and media containing

lipofectamine was mixed together, allowed to sit for 15-20 minutes at room temperature, and added slowly to the well. After 16-18 hours the media was replaced with fresh media.

2.4 J774 Cell Transfection:

J774 macrophages were transfected according to AfCS (Alliance for Cellular Signaling gateway) protocol of *Transfection of Raw Cells*. 1 x 10^5 cells/ml were plated the day before transfection was performed. Most of the reporter assay with J774 cells was done in 24 well plates. For each transfection, 1µl Lipofectamine 2000 was added to 25 µl Opti-MEM in a 1.8-ml Eppendorf tube. In another tube the required amount of plasmid was added, mixed, and allowed to sit for 5 min at room temperature. Then, 25 µl DNA mixtures was added to the 25 µl Lipofectamine 2000 mixture and mixed by tapping or pipetting. This mixture was incubated for 20 min at room temperature. RAWGM1 was added to each well to cover the surface and the plasmid/lipofectamine media was then added. After 3 hours the media was changed and replaced with fresh RAWGM1 media.

RAWGM1 media composition: for 500 ml

DMEM	435 ml	Final concentration 0.87X
FBS	50ml	Final concentration 10%
HEPES	10ml	Final concentration 20mM
L-Glutamine	5ml	Final concentration 2mM

2.5 Immunoprecipitation and Western Blot:

Unless indicated otherwise, we typically transfected 6–10 million 293T cells with the plasmids indicated, using the Lipofectamine 2000 method. Forty-eight hours after transfection, cells were

lysed using a lysis buffer. A quantity of 50–100 µg of total soluble extract was incubated with an appropriate amount of beads conjugated with the specified antibody for 2 h at 4°C. Alternatively, the antibody used for IP was added and kept in a rotating condition for 3-4 hrs and then Agarose A beads were added and incubated overnight. Next day, after washing, the bound proteins were eluted and then separated using SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane (Biorad) and incubated with the specified primary antibody followed by incubation with a secondary antibody conjugated with horseradish peroxidase. The ECL substrate was then added and the blot was developed.

Lysis Buffer: 5mM p Nitrophenyl phosphate, 1% NP-40, 1mM Sodium orthovanadate, 20mM β-glycerophosphate, 1.5mM $MgCl_2$, 150mM NaCl, 50 mM HEPES pH-7.9.

2.6 Generation of stable 293T cells expressing Flag tagged TLR4:

Ten million 293T cells were transfected with 3µg pCL-10A1 and 3µg pMIR-DFT-TLR4 with Lipofectamine 2000. pCl-10A1 is a packaging vector which is used to maximize recombinant retrovirus titers in experimental systems. 48 hrs after transfection virus was collected from the filtered supernatant and spin infection was performed in 293T cells for 90 minutes in presence of 8mg/ml polybrene and 1mM HEPES pH 7.55. The medium was changed after every 2 days and puromycine 1µg/ml was added to select the stable cells. After 2 weeks cells were transfected with required DNA to perform Western Blot and Immunoprecipitation.

2.7 Reporter assay:

For the luciferase reporter assay, 0.25 million 293T cells/well were seeded on 24-well plates. The next day, cells were transfected with Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. A prespecified amount of DNA was added into each

transfection and pcDNA3 DFT empty plasmid was used as filler DNA. When indicated, cells were treated with various stimuli for the specified time. Cells were harvested between 24 and 48 hrs after transfection and luciferase activity was measured using the Luciferase Assay System (Promega). Cells were plated in 24-well plates and transfected with 0.1 μg of IFN-β luc, 0.1 μg κβ –luc and 0.05 μg Renilla luciferase as internal control. Twenty-four hours later, the cells were treated with 0.2 μg/ml LPS, or 40 μg/ml Poly I:C, for 12-16 hrs. The cells were lysed, and luciferase activities were determined using reagent from Promega. The data presented are the mean ± the standard deviation (SD).

2.8 Antibody used:

Antibody to Flag (anti-FLAG) and anti flag beads for IP, anti-HA and anti-β-actin were purchased from Sigma. Anti GFP and Poly(I:C) were purchased from Invitrogen. MG-132 was purchased from Sigma.

2.9 Flow Analysis:

Cells were transfected with YFP-TLR4 and YFP-TRIF plasmid, and also with different concentrations of PLIC-1 and TRIAD 3A plasmid, before the expression of TLR4 and TRIF was observed by flow cytometry. 48 hours after transfection, cells were washed twice with cold PBS and fixed with 10% paraformaldehyde. Flow analysis was done in Epics-XL (Beckman Coulter, Miami, FL) and the data was analyzed with FlowJo software.

3.0 RESULTS

We hypothesized that the PLIC-1 may have a role in the TLR4 signaling pathway. To prove this hypothesis, our objectives were :

AIM 1: Biochemical study of the interaction of TLR4 with PLIC-1.

AIM 2: Biological function of the interaction of TLR4 with PLIC-1 in an over expression system.

AIM 3: Biological function of the interaction of TLR4 with PLIC-1 in loss of function study.

3.1 Aim 1 Results

The results are presented in three sections. Section I describes the biochemical study of the interaction of TLR4 and PLIC-1.

3.1.1 Biochemical study of the interaction of TLR4 and PLIC-1:

Since the interaction of PLIC-1 with the intracellular part of TLR4 has already been confirmed by Yeast Two-hybrid methods, the validation of this interaction was performed by co-immunoprecipitation. As the expression of TLR4 in transient transfection tends to form aggregates and the expression level is also low, a stable cell line based on the cell line 293T was generated expressing Flag-TLR4. To generate this stable cell line 293T cells were transfected with retroviral construct pLC-10A1 and cells were further selected in presence of the antibiotic puromycine. 293T cells stably expressing Flag-TLR4 were transfected with increased concentrations of PLIC-1. The stable line was transfected with 1μg and 5μg of HA-PLIC-1, and IP was subsequently done against anti Flag to pull down Flag-TLR4 and western blot was conducted against Flag antibody (Sigma, 1:500 dilution) to detect pulled-down Flag -TLR4 .The same blot was stripped and probed against HA antibody to detect the expression of PLIC-1. Fig 1A shows the IP to pull down TLR4, and Fig 1B shows HA PLIC-1 co-precipitated with TLR4, indicating an interaction between TLR4 and PLIC-1

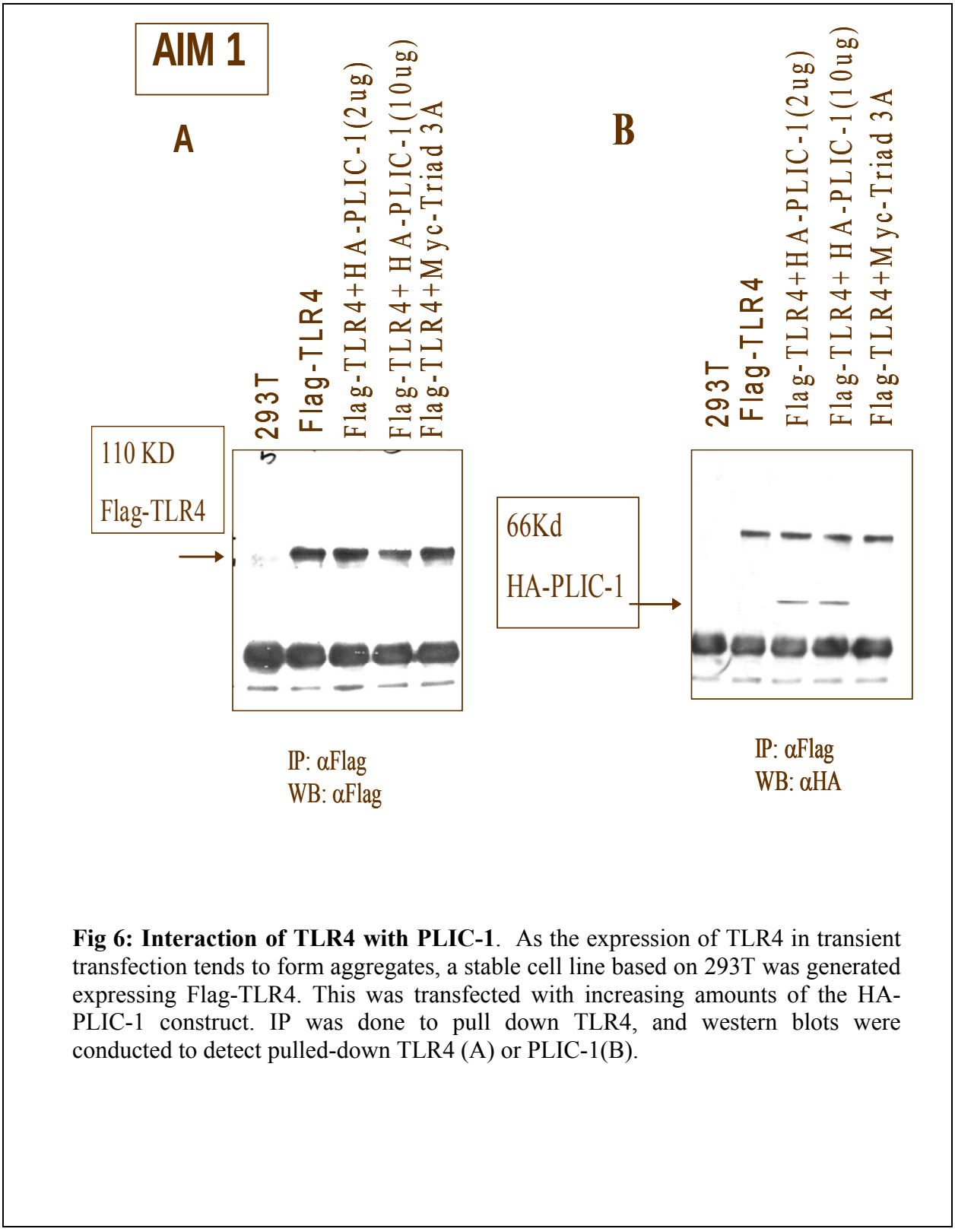


Fig 6: Interaction of TLR4 with PLIC-1. As the expression of TLR4 in transient transfection tends to form aggregates, a stable cell line based on 293T was generated expressing Flag-TLR4. This was transfected with increasing amounts of the HA-PLIC-1 construct. IP was done to pull down TLR4, and western blots were conducted to detect pulled-down TLR4 (A) or PLIC-1(B).

3.1.2 Structure of PLIC-1:

All members of PLIC-1 have an amino terminal Ubiquitin-like domain (Ubl) and carboxy terminal ubiquitin associated domain (Uba) as well as two internal repeats of ~85 amino acids that contain *Sti* I motifs. PLIC-1 is known to bind to membrane proteins including presenilins and GABA receptors. PLIC-1 showed a predominantly cytoplasmic localization. The Uba domain of PLIC-1 was reported to bind with ubiquitylated proteins while the Ubl domains bind ubiquitin ligases like E6AP and βTRCP, and proteosome subunits. These interactions are thought to interfere with normal targeting of proteins for proteosome-dependent-degradation, resulting in enhanced stability potential affecting the signaling pathways in which these proteins are involved

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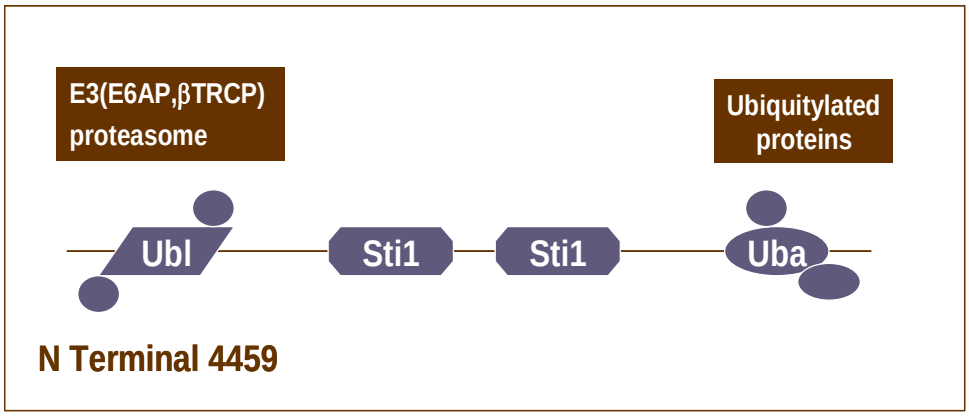


Fig 7: Schematic diagram of PLIC-1 All members of the PLIC-1 has amino terminal ubiquitin like domain (Ubl) and carboxy terminal ubiquitin associated domain (Uba) and two internal motifs that contain Sti 1 motifs. The Ubl domain of PLIC-1 binds to ubiquitin ligases and proteosome subunits. These interactions are thought to interfere with normal targeting of protein for proteosome dependent degradation.

3.1.3 Domain mapping study:

A domain mapping experiment was performed to determine which domain of PLIC-1 is interacting with TLR4. 293T cells were transfected with 5µg YFP-TLR4 and 5µg of N terminal PLIC-1 (4459), full length PLIC-2 (4456) construct, and with full length Flag-PLIC-1 and YFP-TLR4. The expression of flag construct was observed in the input against Flag antibody (Fig A). The pulled-down YFP-TLR4 was observed but when the same blot was stripped against Flag it shows that (Fig C) N terminal of PLIC-1 interacts with TLR4.

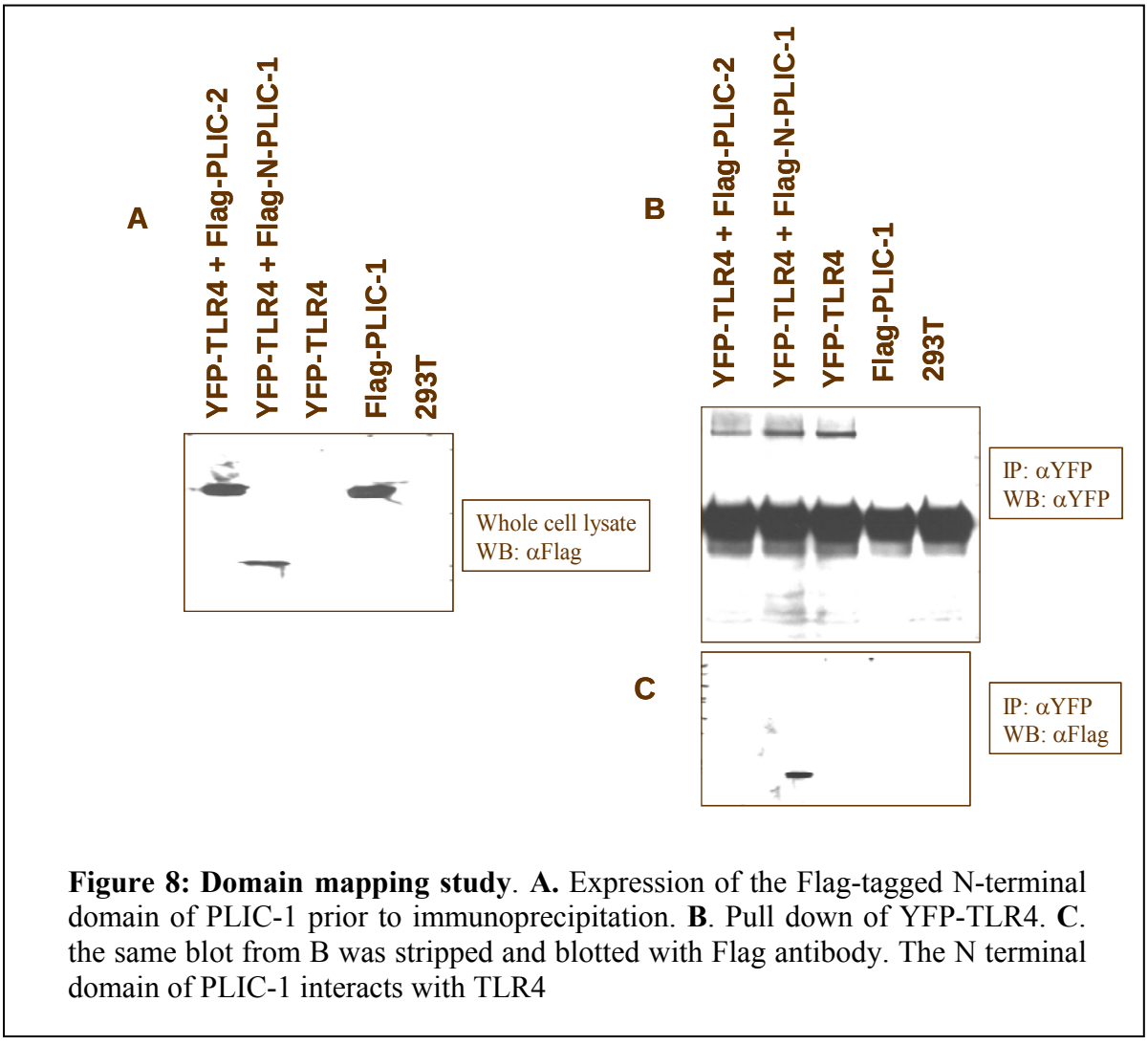


Figure 8: Domain mapping study. **A.** Expression of the Flag-tagged N-terminal domain of PLIC-1 prior to immunoprecipitation. **B**. Pull down of YFP-TLR4. **C**. the same blot from B was stripped and blotted with Flag antibody. The N terminal domain of PLIC-1 interacts with TLR4

3.1.4 The effect of ubiquitination of overexpression of PLIC-1 on the ubiquitination of TLR4:

To analyze whether PLIC-1 modulates the expression of TLR4, cells were transfected with hFc TLR4, HA tagged ubiquitin and with YFP-PLIC-1. HA ubiquitin will be eventually incorporated in all ubiquitinated protein including TLR4. 50μM MG132 was added after 40 hrs of transfection and kept for 6 hrs. MG132 is an inhibitor of proteoseome dependent degradation of proteins which block degradation of polyubiquitinated protein. So in presence of polyubiquitinated protein MG132 will accumulate more ubiquitinated protein which will be easily detectectable by Western blot method against anti HA antibody. After 6 hrs, cells were lysed and western blot was performed against HA antibody with the total cell lysate. It was observed that overexpression of PLIC-1 accumulated more ubiquitinated protein including TLR4. Lane 1 and 2 shows that where HA-UB is absent, no ubiquitinated band was observed. Lane 3 was compared with lane 5. In lane 5 more accumulation of ubiquitinated protein was observed due to the presence of PLIC-1. Lane 4 was compared with lane 6 where the same phenomenon was observed in presence of MG132 that overexpression of PLIC-1 accumulating more ubiquitinated protein including TLR4.

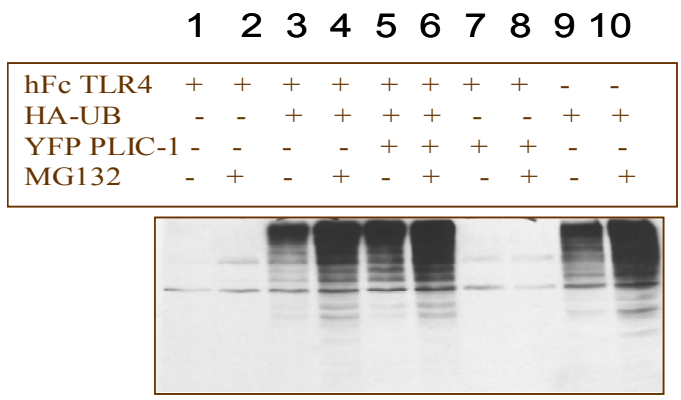


Fig 9: The effect of ubiquitination on overexpression of PLIC-1 on TLR4

3.1.5 The effect of overexpression of PLIC-1 on the ubiquitination of TLR4:

To analyze the effect of overexpression of PLIC-1 on the ubiquitination of TLR4, 293T cells were transfected with YFP-TLR4, HA tagged ubiquitin and FLAG tagged PLIC-1. MG132 was added after 40 hrs of transfection and kept for 6 hrs. After 6 hrs cells were lysed and IP was performed against GFP, and western blot was done against HA antibody. Western blot was done against the IP product as well as total cell lysate. IP was done against the samples where MG132 was added as MG132 will accumulate more ubiquitinated protein which will be easily detectectable by Western blot method against anti HA antibody. But in case of whole cell lysate Western Blot was performed against sample where MG132 is present as well as well as absent. In case of IP (left figure) lane 1 was compared with lane 2 where it was observed that overexpression of PLIC-1 accumulated more ubiquitinated TLR4. TRIAD3A is a positive control and it is known that overexpression of TRIAD3A accumulates more ubiquitinated TLR4. In case of total cell lysate (right figure) lane 3 was compared with lane 5, and lane 4 with lane 6. In each case it was observed that overexpression of PLIC-1 accumulated more ubiquitinated protein.

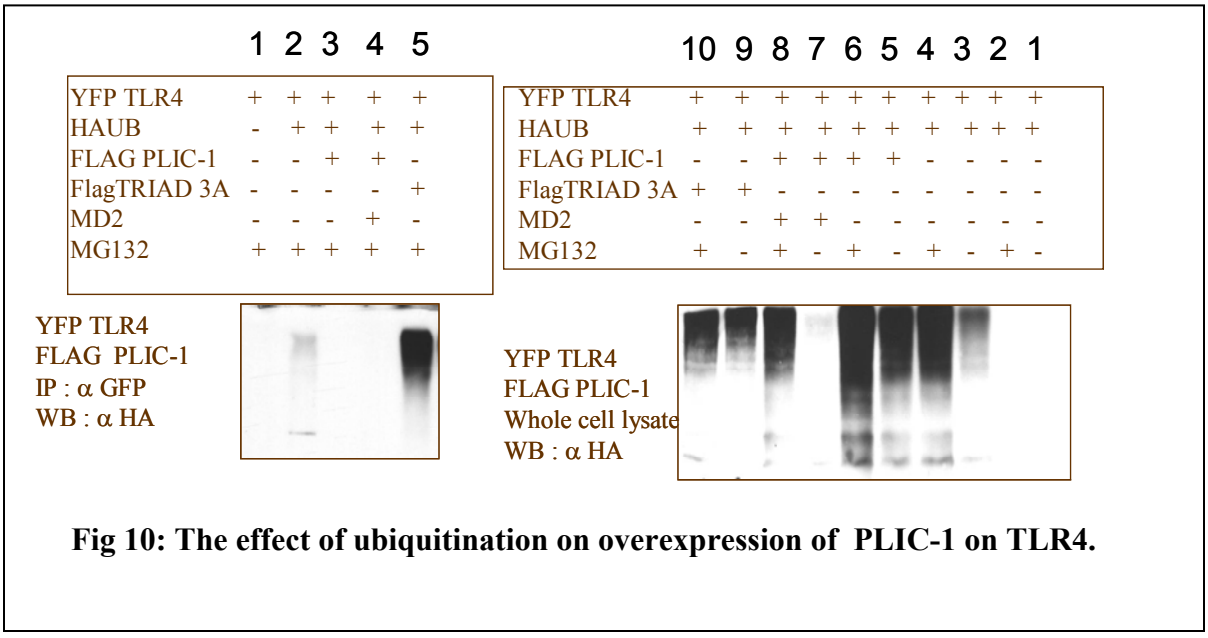


Fig 10: The effect of ubiquitination on overexpression of PLIC-1 on TLR4.

3.1.6 The effect of PLIC-1 on TLR4 by flow analysis:

Flow analysis was performed to analyze the effect of overexpression of PLIC-1 on TLR4. 293T cells were transfected with 0.5ug YFP-TLR4 and 0.1, 0.2, 0.4, or 0.8µg of PLIC-1. 293T cells without any transfected DNA was used as negative control. It was observed that in presence of increasing amounts of PLIC-1 the expression of TLR4 was also increased. So overexpression of PLIC-1 is stabilizing TLR4.

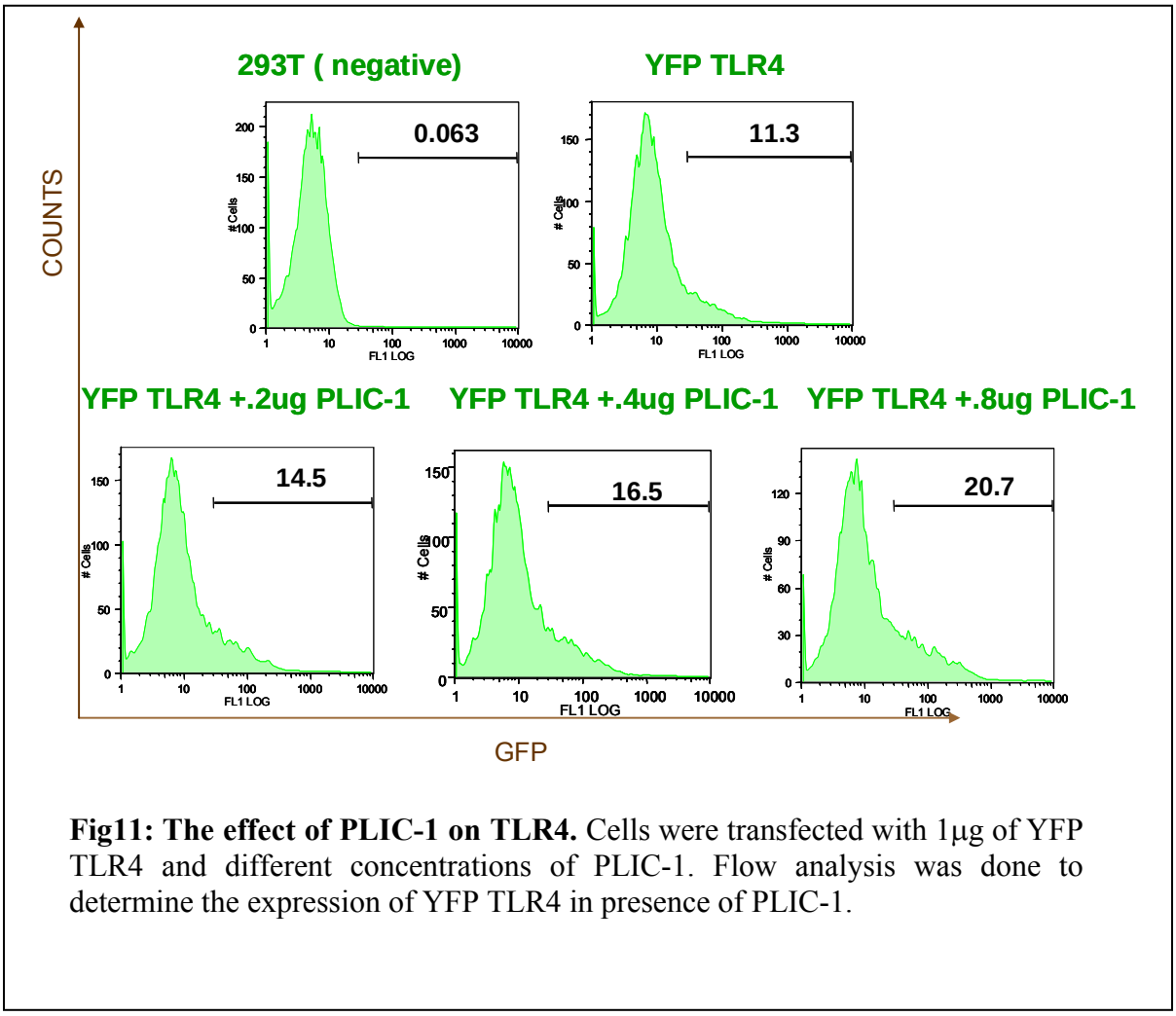


Fig11: The effect of PLIC-1 on TLR4. Cells were transfected with 1µg of YFP TLR4 and different concentrations of PLIC-1. Flow analysis was done to determine the expression of YFP TLR4 in presence of PLIC-1.

3.2 Aim 2 Results

AIM 1: Biochemical study of the interaction of TLR4 with PLIC-1.

AIM 2: Biological function of the interaction of TLR4 with PLIC-1 in an over expression system.

AIM 3: Biological function of the interaction of TLR4 with PLIC-1 in loss of function study.

3.2.1. Effect of NS3/4A, PLIC-1 and Sars 3C protease in TRIF dependent signaling pathways:

Akira et al (2001) reported that TLRs activate the transcription factors NF-κB and AP1, leading to production of inflammatory cytokines such as tumor necrosis factor (TNF-α) and up-regulation of the co-stimulatory molecules CD80 and CD86 on dendritic cells (DCs)[48]. Yamamoto (2002)[51] identified a novel TIR domain-containing molecule, named TIR domain-containing adapter (TRIF), and also showed that, like MyD88 and TIRAP, overexpression of TRIF activated the NF-κβ-dependent promoter. Several reporter assays were performed to find out the function of PLIC-1 on TRIF dependent signaling. In the first experiment 293T cells were transfected with 0.1μg of IFN-β luc reporter construct and 0.5μg of renilla luciferse as internal control. Then cells were also transfected with 0.1μg and 0.2μg NS3/4A (HCV protease), Sars 3C protease and PLIC-1. After 24 hrs of transfection, cells were again transfected with 0.5μg of TRIF. After 48 hrs cells were lysed and a reporter assay was performed. It was observed that NS3/4A, which is known to degrade TRIF, inhibited TRIF dependent IFN-β activation in a dose dependent manner. The same result was observed in case of PLIC-1 and Sars 3C protein (Fig 12 A). To rule out the possibility of promoter interference and squelching effects of between IFN-β and PLIC-1, construct cells were transfected with different concentrations of PLIC-1 plasmid and 0.1μg of IFN-β luc reporter plasmid. After 48 hrs the cells were lysed and a reporter assay was performed. We observed that that in absence of TRIF only PLIC-1 is able to enhance IFN-β luc production (Figure 12B) which rules out the possibility that the inhibition of TRIF dependent IFN-β activation in presence of PLIC-1 is not due to promoter interference or squelching effects.

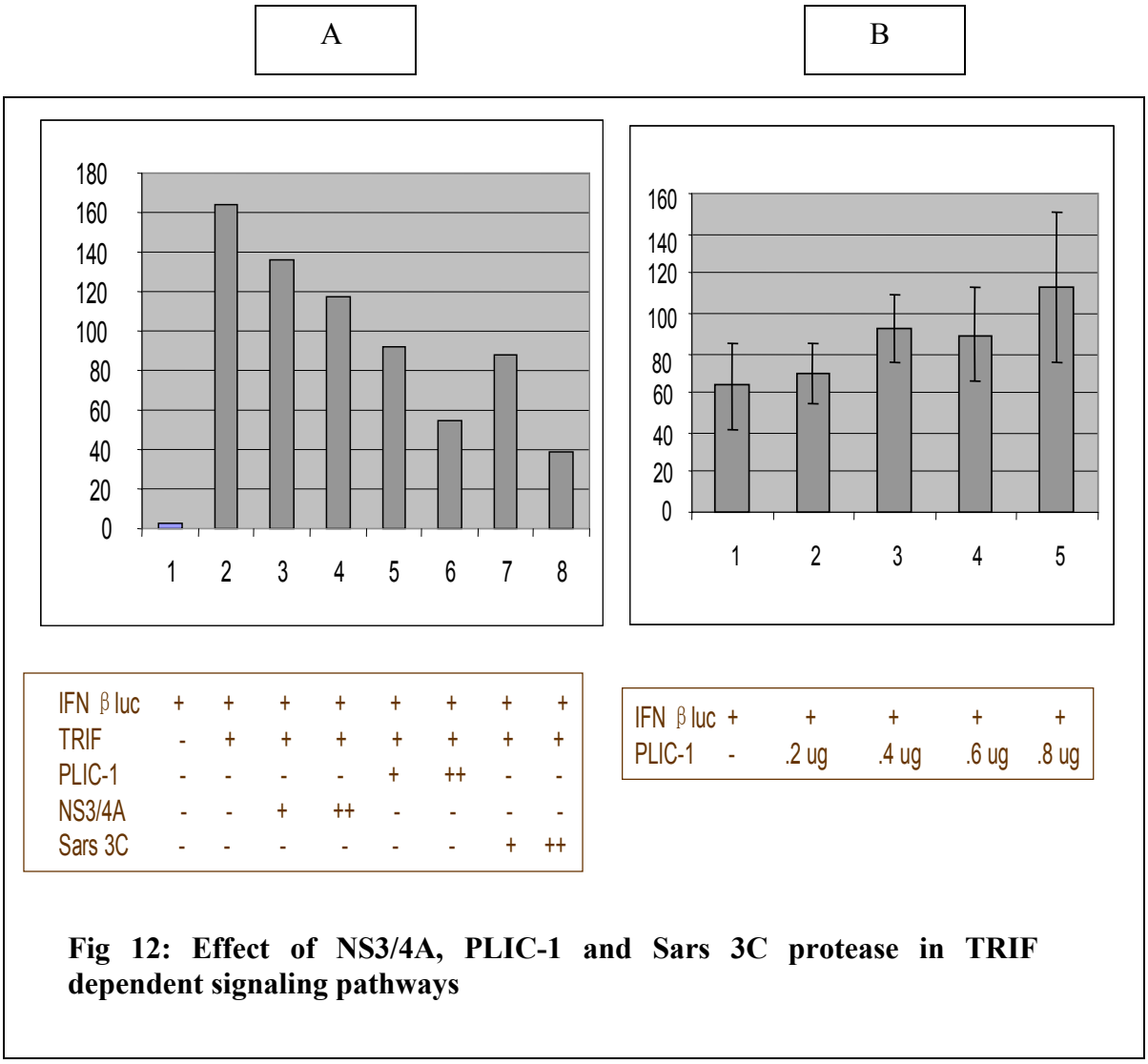


Fig 12: Effect of NS3/4A, PLIC-1 and Sars 3C protease in TRIF dependent signaling pathways

3.2.2 Over expression of PLIC-1 on the TRIF dependent IFN-β pathway:

To observe the effect of over expression of PLIC-1 in TRIF dependent on IFN-β pathway, cells were transfected with 0.1μg of IFN-β luc reporter construct and 0.5μg of Renilla luciferase as internal control and 0.2, 0.4, 0.6, 0.8 μg of HA-PLIC1 (Figure 13A), YFP-PLIC-1 (Figure 13B) and Flag-PLIC-1 (Figure 13C) and the production of IFN-β luc was measured by reporter assay. Cells were transfected with each construct in the same day and after 24 hrs of transfection the cells were again transfected with 0.5μg of TRIF. After 48 hrs cells were lysed and the reporter

assay was performed. It was observed in all three individual cases that the presence of high concentrations of PLIC-1 inhibits IFN-β luc. In the case of HA-PLIC-1 with the highest concentration (0.8µg of HA-PLIC-1) of HA- PLIC-1 5 fold inhibition in the production of IFN-β, in case of YFP-PLIC-1, 8 fold and in case of Flag PLIC-1 5 fold inhibition was observed. Data was normalized by using renilla luciferase as an internal control.

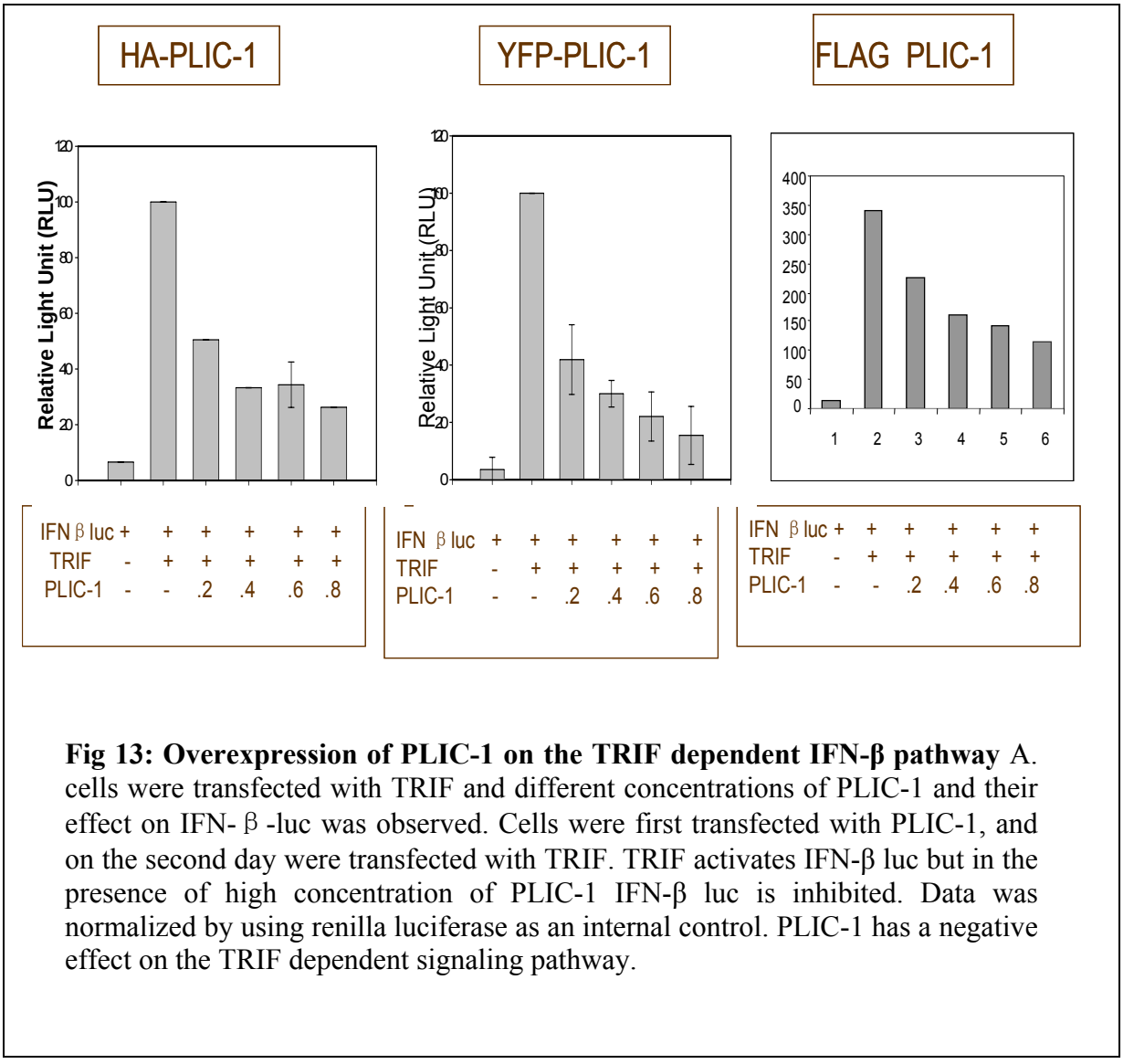


Fig 13: Overexpression of PLIC-1 on the TRIF dependent IFN-β pathway A. cells were transfected with TRIF and different concentrations of PLIC-1 and their effect on IFN- β -luc was observed. Cells were first transfected with PLIC-1, and on the second day were transfected with TRIF. TRIF activates IFN-β luc but in the presence of high concentration of PLIC-1 IFN-β luc is inhibited. Data was normalized by using renilla luciferase as an internal control. PLIC-1 has a negative effect on the TRIF dependent signaling pathway.

3.2.3 The effect of overexpression of PLIC-1 on the TRIF dependent κB activation:

As Yamamoto (2002) showed, like MyD88 and TIRAP, over expression of TRIF activates the NF-κB-dependent promoter. To observe the effect of over expression of PLIC-1 on the TRIF dependent NF-κB pathway, cells were transfected with 0.1µg of κB luc reporter construct, with 0.5µg of Renilla luciferase as an internal control, and 0.2, 0.4, 0.6, or 0.8µg of YFP-PLIC. Cells were first transfected with PLIC-1 and on the second day were transfected with TRIF. After 48 hrs cells were lysed and the reporter assay was performed. TRIF activates κB-luc but it was observed that, in the presence of high concentration of PLIC-1, TRIF dependent activated κB-luc is inhibited.

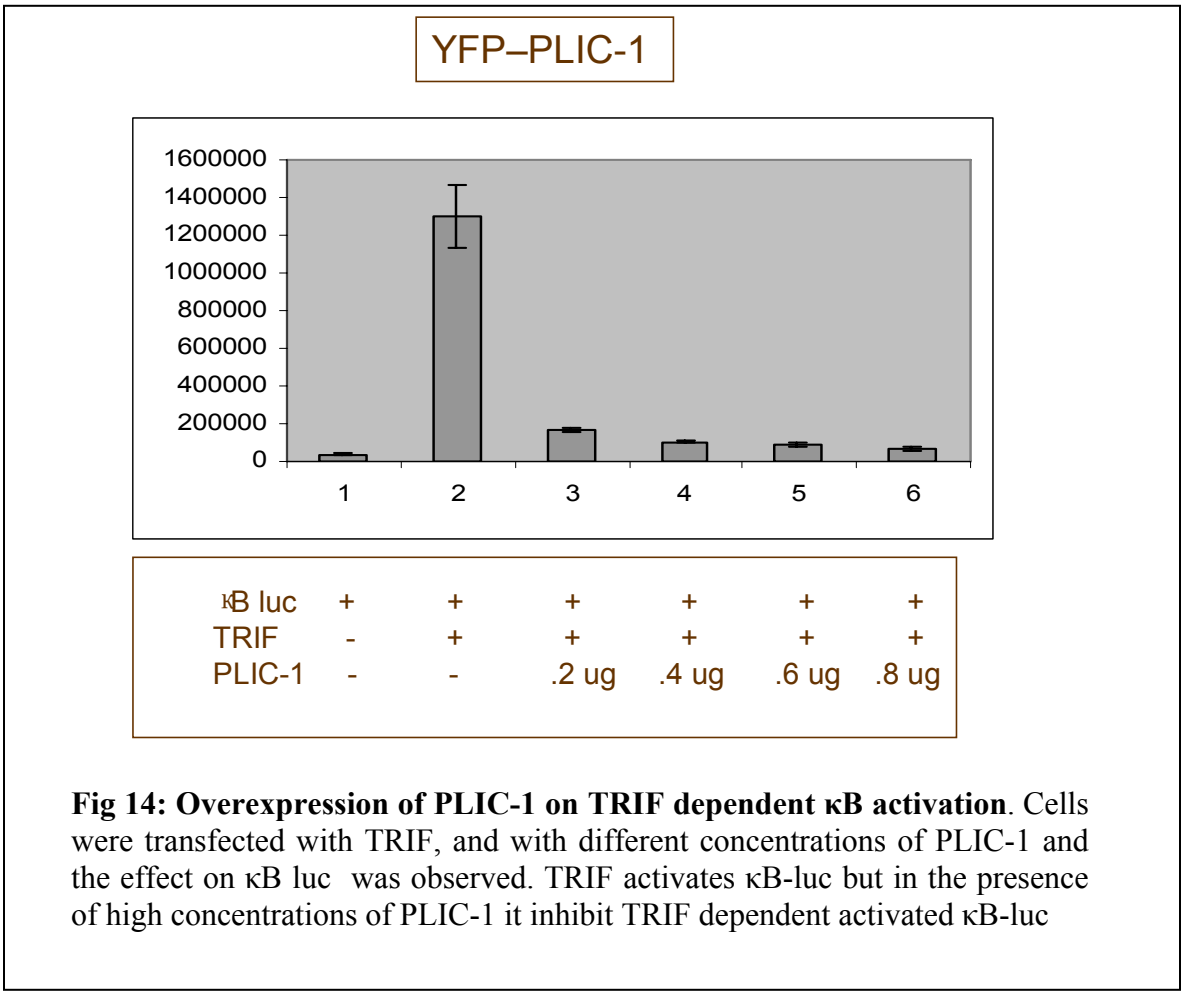


Fig 14: Overexpression of PLIC-1 on TRIF dependent κB activation. Cells were transfected with TRIF, and with different concentrations of PLIC-1 and the effect on κB luc was observed. TRIF activates κB-luc but in the presence of high concentrations of PLIC-1 it inhibit TRIF dependent activated κB-luc

3.2.4 Effect of PLIC-1 on TRIF by Flow analysis:

Flow analysis was performed to further support the notion that PLIC-1 is degrading TRIF, and eventually inhibiting the production of IFN-β. 293T cells were transfected with 1µg of YFP-TRIF and with 0.5µg, 1µg, 1.5µg or 2µg of HA PLIC-1, and the expression of TRIF was observed by flow analysis. TRIAD3A, which is known to degrade TRIF, was used as a positive control. 293T cells were transfected with 1ug of YFP-TRIF and 0.5µg, 1µg, 1.5µg or 2µg of myc tagged TRIAD3A, and the expression of TRIF was observed by flow analysis. It was observed that increased amount of PLIC-1 decreased TRIF protein.

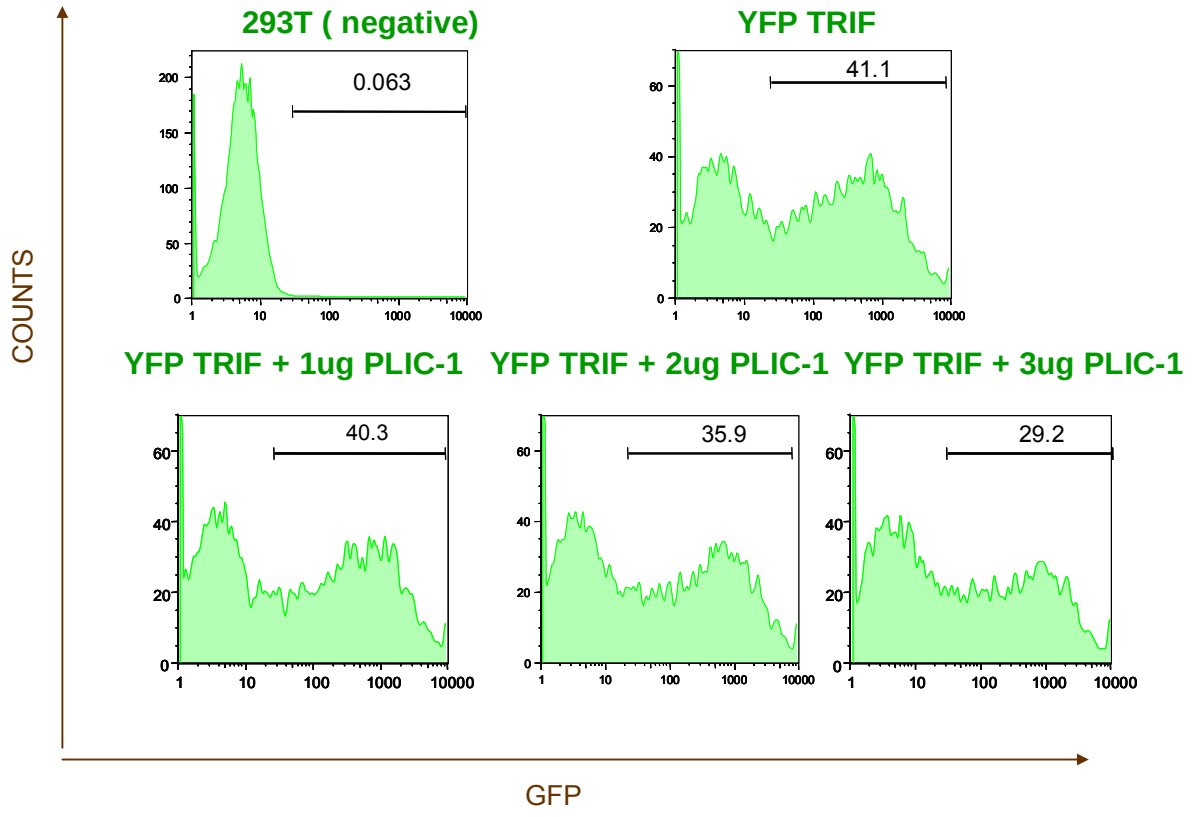


Fig 15 A: The effect of PLIC-1 on TRIF. Cells were transfected with1ug of YFP TRIF and different concentration of PLIC-1 and flow analysis was done to determine the expression of YFP TRIF in presence of PLIC-1.

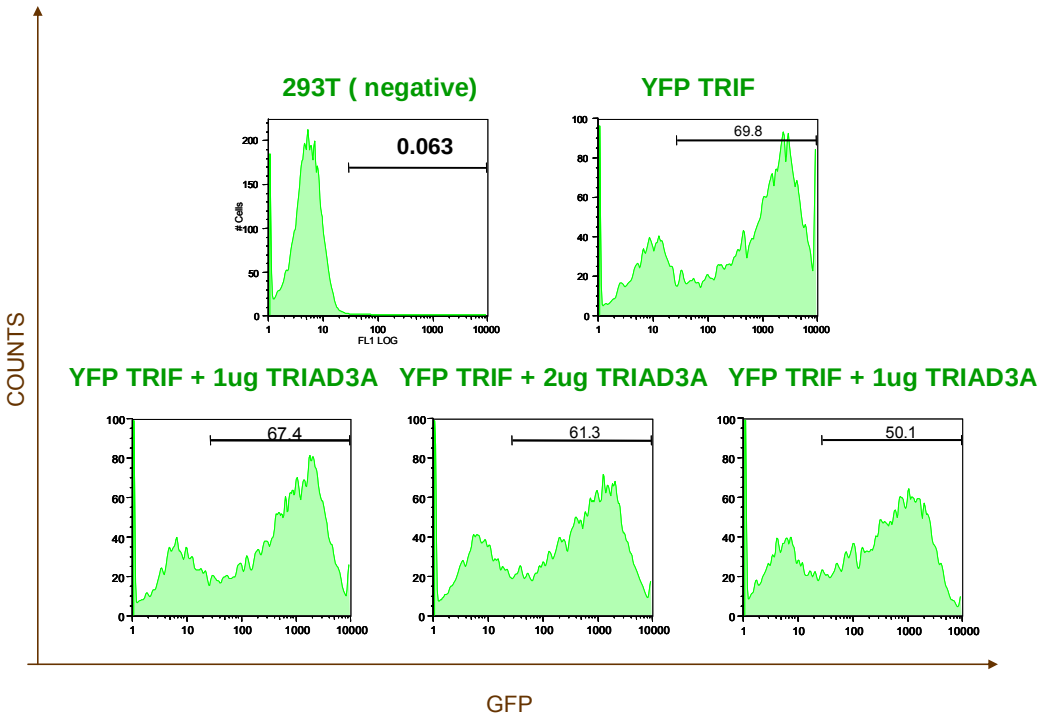


Fig 15 B: The effect of TRIAD3A on TRIF. Cells were transfected with 1ug of YFP-TRIF, and different concentrations of PLIC-1, and flow analysis was used to determine the expression of YFP TRIF in presence of TRIAD3A.

3.3 Aim 3 Results

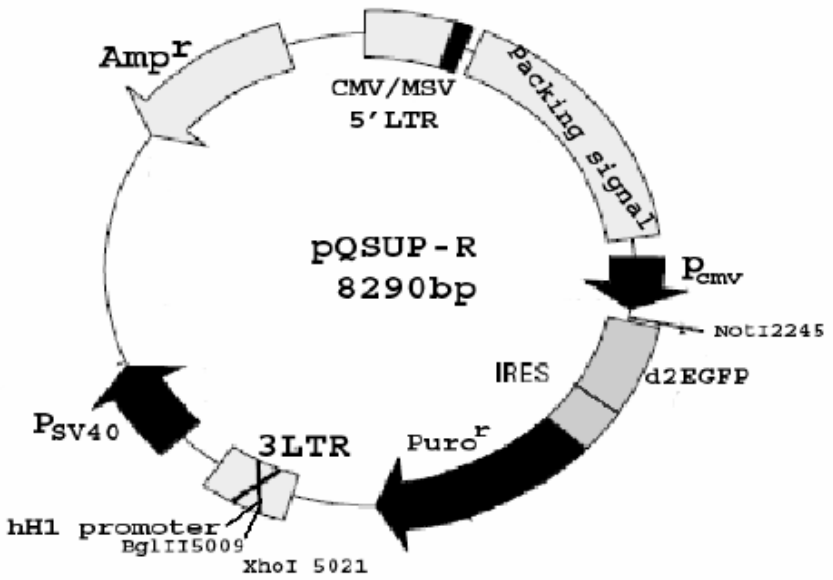
AIM 1: Biochemical study of the interaction of TLR4 with PLIC-1

AIM 2: Biological function of the interaction of TLR4 with PLIC-1 in an over expression system

AIM 3: Biological function of the interaction of TLR4 with PLIC-1 in loss of function study.

3.3.1 RNAi vector and sequence:

RNA interference (RNAi), also known as post transcriptional gene silencing, is mediated by either small interfering RNA (siRNA) or by micro RNA (miRNA). Here, we used different vector based RNAi. In these experiments, RNAi was used to knock down endogenous expression of PLIC-1. The vector backbone of the RNAi, and the position in PLIC-1 it targets, are shown below. The vector has a chloramphenicol selection marker as well as a GFP tag which helps to select the transfected cells and is useful for making stables. Four human RNAi, targeting the positions 1602 and 733, and 819, hLMP and one mouse RNAi targeting endogenous PLIC-1 were tested.



PLIC-1 (1602) GCACAGATGATGCTGAATAAT

PLIC-1 (733) GCAATGTTACTACATCATCAA

PLIC-1 (819) gcgcatgtacacagatattca

Fig 16: RNAi vector and sequence: The sequences in the red are the targeting sequence.

3.3.2 Working RNAi against human PLIC-1 and the working dose of RNAi required:

Western blot analysis was performed to find out which RNAi is working best among the four RNAi against endogenous human PLIC-1, and to determine the minimum concentration of RNAi that is able to knock down endogenous PLIC-1. Cells were transfected with 0.1μg of HA tagged human PLIC-1 and either 0.1μg or 2μg of RNAi 1602, 733, 819, and another RNAi against human PLIC-1, hLMP. After 48 hours cells were lysed and the western blot was performed against anti HA monoclonal antibody. (Fig 17A). RNA I again endogenous mouse PLIC-1 was used as a negative control where 293T cells were transfected with 0.1μg of PLIC-1 and different concentration, (0.1μg, 1μg, 2μg, 3μg, 4μg, and 5μg) of mouse RNAi against endogenous PLIC-1. It was observed that RNAi 819 worked best although 1602 also worked equally well. In the case of 1602, 2ug of the RNAi was able to completely knock down endogenous PLIC-1. Here, mLMP RNAi against endogenous mouse PLIC-1 was used as a negative control and no effect was observed in that case as the PLIC-1 was against human PLIC-1. Data was normalized with endogenous actin to show the loading was equal for each sample.

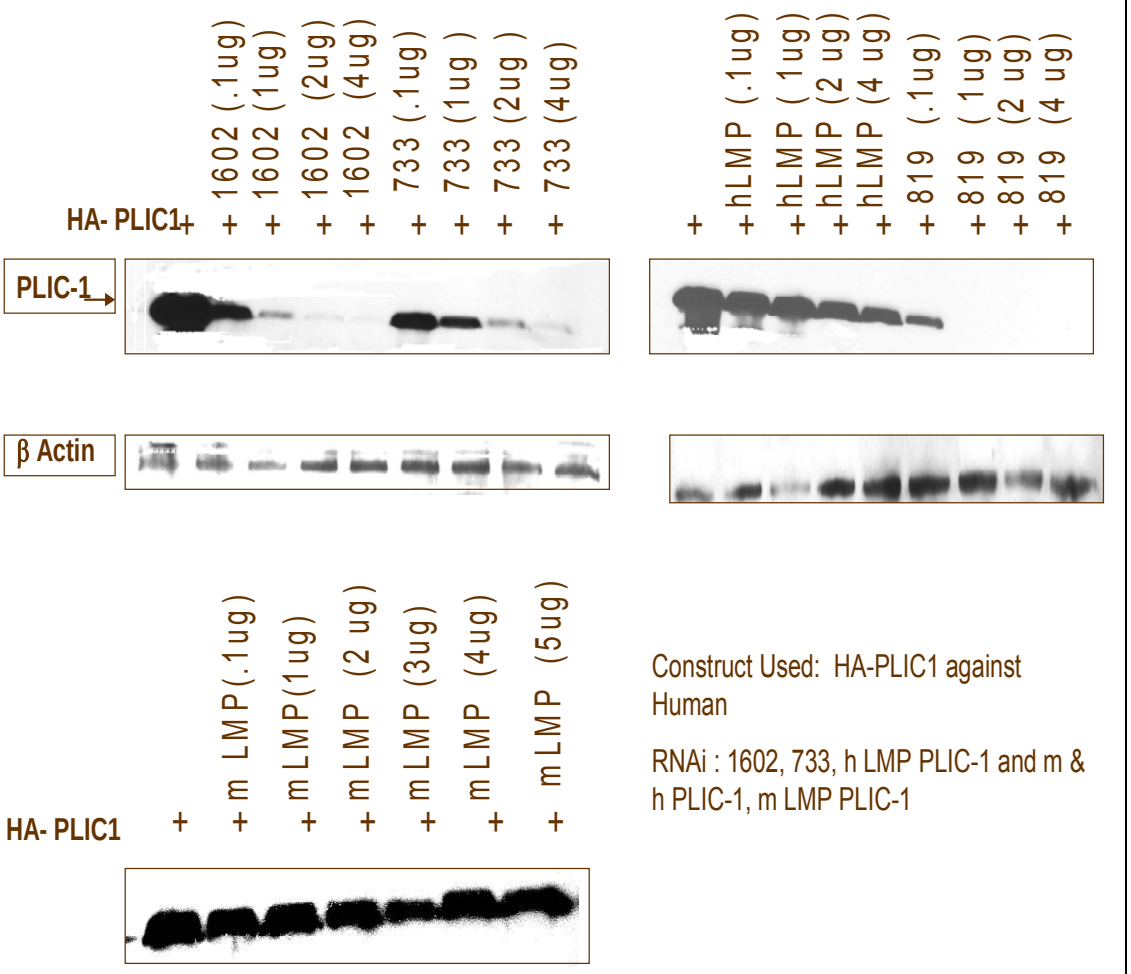
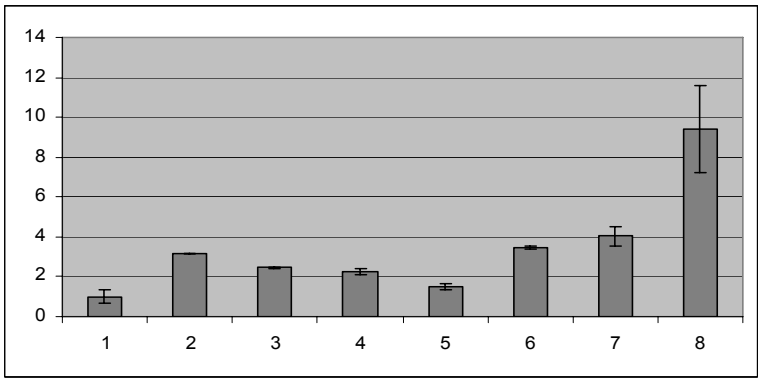


Fig 17: **Working RNAi against PLIC-1 and the working dose required.** To find out the best RNAi against PLIC-1, and the working dose, cells were transfected with 0.1µg of HA-PLIC1 and then transfected with different concentrations of RNAi. It was observed that RNAi 1602 worked better although 819 worked best and, in these two cases, the RNAi effect was observed even in lower doses. The specificity was determined by transfecting the cells with mLMP PLIC-1 which is specific for mouse. Inhibition was not observed in this case.

3.3.3 Effect due to the knockdown of endogenous PLIC-1 in TRIF dependent signaling pathways:

After identifying the optimal RNAi construct and working dose, the effect of knockdown of endogenous PLIC-1 on the TRIF-dependent signaling pathways was determined. 293T cells were transfected with 0.1µg of IFN-β luc reporter construct and 0.5µg of Renilla luciferase as internal control, as well as with 0.2, 0.4, and 0.6µg of HA-PLIC1 and 1602 RNAi. Cells were transfected with all of the constructs on the same day. After 24 hrs of transfection the cells were again transfected with 0.1µg of TRIF. After 48 hrs, the cells were lysed and the reporter assay was performed. It was observed that overexpression of PLIC-1 inhibits activation of TRIF dependent IFN-β luc, but due to the knock down of endogenous PLIC-1 with RNAi, TRIF-dependent IFN β luc was further induced.



IFN β luc	+	+	+	+	+	+	+	+
TRIF	-	+	+	+	+	+	+	+
HA-PLIC-1	-	-	+	++	+++	-	-	-
1602	-	-	-	-	-	+	++	+++

+ .1 ug

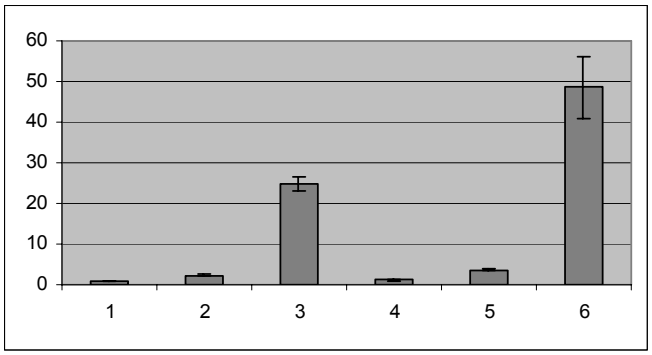
++ 1 ug

+++ 1.5ug

Fig 18: Effect due to knockdown of endogenous PLIC-1 in TRIF dependent signaling pathways. Cells were transfected with differing concentrations of HA-PLIC1 and RNA-I 1602 against PLIC-1. It was observed that overexpression of PLIC-1 inhibits activation of TRIF dependent IFN-β luc, but due to the knock down of endogenous PLIC-1 with RNAi, TRIF-dependent IFN β luc was further induced.

3.3.4 Effect of PLIC-1 in TRIF-dependent TLR3 signaling pathways:

As TLR3 signaling is TRIF-dependent a reporter assay was performed to determine whether PLIC-1 inhibits TRIF dependent IFN- β production and also to show whether it has any effect on TLR3-associated pathways. 293T cells were transfected with 0.1μg of IFN-β luc reporter construct and 0.5μg of Renilla luciferase as internal control, as well as with 0.1μg TLR3 plasmid and RNAi 1602. After 24 hours, cells were stimulated with 40 μg/ml Poly I: C for 16 hours and after 48 hours cells were lysed and reporter assay was performed. It was observed that due to the knock down of endogenous PLIC-1 with RNAi, TRIF-dependent IFN β luc was further induced.



IFN- β luc	+	+	+	+	+	+
TLR3	-	+	+	-	+	+
1602	-	-	-	+	+	+
Poly IC	-	-	+	-	-	+

Fig 19: Effect of PLIC-1 in TRIF dependent TLR3 signaling pathways. A. cells were transfected with TLR3 and RNAi against human endogenous PLIC-1 and IFN-β luc production was measured. As TLR3 signaling is TRIF-dependent, depletion of PLIC-1 induces TRIF-dependent IFN-β luc. Poly I:C was used to stimulate the cells as it is a ligand of TLR3. Data were normalized by using renilla luciferase as an internal control.

3.3.5 The effect of overexpression of PLIC-1 on the TRIF-dependent IFN-β pathway in macrophages:

To observe the effect of PLIC-1 in macrophages, the mouse macrophage J774 was transfected with PLIC-1 and the production of IFN-β was analyzed by reporter assay. Cells were transfected with 0.5µg of IFN-β luc reporter construct and 0.5µg of Renilla luciferase as internal control, as well as with and 0.2, 0.4, 0.6, and 0.8µg of YFP PLIC-1. After 24 hours cells, were stimulated with poly I:C (40 µg/ml), which is used to stimulate the production of interferon by the immune system. After 48 hrs cells were lysed in 100µl of lysis buffer and reporter assay was performed. All transfections were done in 24 well plates. It was observed that overexpression of PLIC-1 inhibits activation of TRIF dependent IFN-β luc.

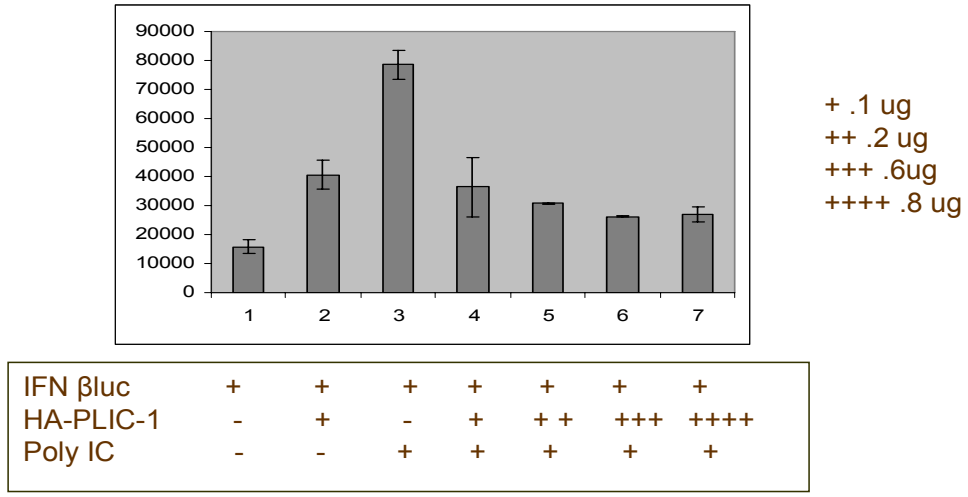


Fig 20: Overexpression of PLIC-1PLIC-1 in macrophages and its effect on the IFN β -luc pathway. Something is missing here PLIC-1 and the effect on IFN- β -luc was observed. After 24 hrs cells were stimulated with Poly I:C. PLIC-1 activates IFN-β luc overexpression of PLIC-1 inhibits activation of TRIF dependent IFN-β luc. Data was normalized by using renilla luciferase as an internal control.

4.0 DISCUSSION

The PLIC proteins belong to a family of proteins that contain a ubiquitin-like (ubl) domain as an integral part of their open reading frame. Ubiquitin-like (ubl) proteins can be subdivided into two general classes; small-sized type 1 ubl proteins that are covalently linked to target proteins in a fashion similar to ubiquitin, and type 2 ubl proteins that are not ligated to other proteins and whose functions are not yet well understood [52]. This protein is a member of a group of related proteins like PLIC-2. Protein degradation plays an important role in a wide array of cellular events. Eukaryotic cells have evolved two machineries to execute many of the controlled proteolytic events: the ubiquitination machinery and the proteasome. The ubiquitination machinery recognizes and tags specific proteins that are to be destroyed, whereas the proteasome degrades the ubiquitinated substrates. According to Hershko, et al the collaborative action of these two machineries is crucial for a variety of diverse processes including cell cycle progression, development, apoptosis, signal transduction, and antigen presentation [53]. Toll like receptors (TLRs) are critical innate immune receptors that recognize microbial pathogen and trigger the first line of host defense. Several regulatory mechanisms that seem to control TLR signaling have been described, and one of them uses proteins linked to the negative regulation of TLR signaling including IL-1R-associated kinase M (IRAK-M) and the suppression of cytokine signaling [54]. Also, Triad3A acts as an E3 ubiquitin-protein ligase and enhances ubiquitination and proteolytic degradation of some TLRs [55]. Here we used Wedtern blot and

immunoprecipitation to identify PLIC-1 interactions with the cytoplasmic domain of TLR4. After a domain mapping experiment we determined that TLR4 is interacting with the N terminal domain of PLIC-1. Although PLIC-1 has been reported to be involved in protein degradation [56-58], overexpression of PLIC-1 accumulating more ubiquitinated protein including TLR4 and inhibits TLR4 from undergoing proteosomal degradation. As a result flow analysis showed that PLIC-1 stabilizes TLR4. TRIF is the TLR adaptor that characteristically induces IFN-β transcription [59-60]. TRIF is a TIR domain-containing adapter protein that is essential for all signaling by TLR3 and some signaling by TLR4. It was also shown that TRIF was the only one that efficiently induced apoptosis when over expressed in 293T cells [61]. It was observed that overexpression of PLIC-1 inhibits activation of TRIF dependent IFN-β luc as well as NF-κB luc. It was already reported by Tsung-Hsien el al that the RING finger protein Triad3A acts as an E3 ubiquitin-protein ligase, and enhances ubiquitination and proteolytic degradation of some TLRs. Triad3A negatively regulates TLR activation [62]. Our use of a reporter assay with a different PLIC-1 construct confirms that overexpression of PLIC-1 inhibits activation of TRIF dependent IFN-β luc. By flow analysis it was observed increased amount of PLIC-1 decreased TRIF protein. In this experiment TRIAD3A was used as a positive control known to degrade TRIF [62]. From the flow data it was confirmed that PLIC-1 degrades TRIF and eventually inhibits IFN-β as well as NF-κB production. RNAi constructs were made, and the optimal RNAi and amounts required were determined by western blot. RNAi against position 819 and 1602 worked best compared to the others. When the RNAi was used to deplete endogenous PLIC-1, a reporter assay showed that due to the knock down of endogenous PLIC-1 with RNAi, TRIF-dependent IFN β luc was further induced. The same experiment was performed on the mouse macrophage

cell line J774. The same inhibition of TRIF-dependent IFN-β was observed. Taken together, these preliminary data suggest that PLIC-1 is a negative regulator of TRIF-mediated pathways.

5.0 FUTURE WORK

The following works could be performed in the future :

1. Mouse RNAi will be used to make retrovirus and to transduce J774 macrophage cells to knock down endogenous PLIC-1, so that the production of IFN-β could be monitored by ELISA

2. A Phagocytosis assay will be performed to show whether there is any effect in phagocytosis due to the interaction of these two proteins.

3. IP and western blot will be performed to know whether TRIF interacts with PLIC-1.

4. An immunofluorescence study will be performed to show the co-localization of these two proteins.

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